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FINAL PROJECT REPORT

CO_2 CONVERSION BY KNALLGAS MICROORGANISMS

Evaluation of Products and Processes

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PREFACE

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ABSTRACT

Biological capture and converting carbon dioxide (CO_2) into useful organic molecules has traditionally focused on photosynthetic processes provided by plants or microalgae. More recently, an alternative approach under development for the biological capture and converting CO_2 is using microorganisms called chemoautotrophs. Instead of using light (photosynthesis) to power the capture of CO_2 like plants and algae, chemoautotrophs use a range of chemicals such as hydrogen (H_2), metal ions, and sulfides to power the carbon capture reaction. However, approaches applying such chemoautotrophic microorganisms to capturing and converting CO_2 into useful organic chemicals, such as transportation fuels, have received less attention than traditional sugar-based or direct photosynthetic approaches to producing biofuels and chemicals.

Kiverdi is developing a promising class of chemoautotrophic microorganisms, called knallgas microorganisms, which can convert CO_2 into energy-rich oils using H_2 gas. H_2 gas can be generated from a range of renewable, CO_2 emission-free energy sources including solar, wind, and hydroelectric power, as well as from the gasification of biomass. The oils that are produced from CO_2 can be made into transportation fuels like diesel, or converted into other useful products like surfactants and solvents.

The research team developed bioprocesses using knallgas microorganisms grown on CO_2 and H_2 gaseous bases to convert CO_2 into oil and other hydrocarbon products. Laboratory development was used to guide the scale up of bioprocesses to 20 L reactor scale and culminated in the design of a 50 L scale mobile pilot facility that could be used for testing at various potential CO_2 feedstock locations. This report describes the processes, methods, and equipment used to achieve robust growth of a model knallgas microorganism; oil and other biochemical products generated by the organism, and establishes a proof of concept for knallgas bioprocess technology.

Keywords: Biofuel, Biochemical, Carbon Sequestration, Carbon Capture, Knallgas Bacteria, Oxyhydrogen Bacteria, Hydrogenotroph, Chemoautotroph, Chemosynthesis, Syngas Conversion, Gas Fermentation, Gas Bioprocess, Bioprocess, CO_2, Carbon Dioxide, CO_2 utilization, CO_2 use, CO_2 reduction, Greenhouse Gas, GHG, GHG reduction, CO_2-to-oil, CO_2-to-chemical, Waste Conversion, Gas-to-liquids, Gas-to-chemicals, GTL, GTC, Lipid, Triacylglycerol, Triacylglyceride, TAG, Fatty Acid, Fat, Biodiesel, Biojet, Terpene, Hydrocarbon, Biological Oil, Microbial Oil, Oleochemical, Bioeconomy, Biobased chemical, Petrochemical Replacement, Petroleum replacement, Electrofuels, H_2, Hydrogen, Hydrogen economy, Global Warming Solutions Act, AB 32, Low Carbon Fuel Standard, Executive Order B-30-15

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California's Greenhouse Gas Reduction Legislation

In California, the Global Warming Solutions Act (AB 32) requires greenhouse gas (GHG) emissions in the state reduced to 1990 levels by 2020. The Low Carbon Fuel Standard (LCFS), California, Executive Order S-1-07, calls for a reduction of at least 10 percent in the carbon intensity of California's transportation fuels by 2020. More recently, Executive Order B-30-15 establishes a California GHG reduction target of 40 percent below 1990 levels by 2030 - the most aggressive benchmark enacted by any government in North America. These measures reflect a necessity for non-petroleum-based alternative fuels and chemicals that reduce carbon emissions and carbon capture and conversion solutions.

The Need for Alternative Fuels and Chemicals

Petroleum has been a major source of fuels and chemicals for more than a century and is refined into gasoline, diesel and jet fuel - the primary transportation fuels. Petroleum also provides the chemical feedstocks required to manufacture an array of products, including plastics, cosmetics, detergents, lubricants, pharmaceuticals, computer components and thousands of other essential products. There is a necessity for the sustainable, renewable production of these fuels and chemicals from alternative, non-petroleum sources. In addition to alternative sources of these chemicals and fuels, it is also essential to produce these alternatives so they do not compete with food production, cause major disruption in land use or natural habitats, or consume large quantities of fresh water or fertilizer. Although substantial steps have been taken towards clean alternative energy sources for electricity generation, organic molecules, such as hydrocarbons, remain important for chemical and transportation fuel applications, and the demand for many oil-derived products and materials will continue to persist. Despite the projected increase in plug-in hybrids and electric vehicles, and mandated increases to corporate average fuel economy standards, the demand for high energy density, liquid fuels in transportation is projected to remain fairly constant over the next 20 years.

Project Purpose

Turning Waste CO_2 into Useful Products

Kiverdi is commercializing a technology converting carbon dioxide (CO_2) and other carbon-based wastes into products such as oils, industrial chemicals and fuels. Profitably converting CO_2 into useful products creates an incentive to capture it, rather than simply release it into the atmosphere. The chemicals produced from CO_2 could also displace petroleum products, and avoid emissions from their production and use, leading to additional greenhouse gas reductions.

This process uses special types of microorganisms (chemoautotrophs) to capture and convert CO_2 gas into organic molecules. Some types of chemoautotrophs can also convert other gases comprised of molecules having a single carbon atom such as carbon monoxide (CO), syngas (a mixture of H_2, CO, and CO_2), or methane (CH_4). Unlike plants and algae that use light to capture and convert CO_2, these microorganisms use the energy stored in inorganic chemical

molecules. The specific class of chemoautotrophs for this project - knallgas microorganisms - use the energy stored in hydrogen gas (H_2) to drive the biochemical capture and conversion of CO_2 or CO. Knallgas is a mixture of oxygen and hydrogen gases that, in this case, is metabolized by the microorganism during its natural lifecycle. These knallgas microorganisms use the energy derived from the reaction of H_2 and O_2 to drive the chemical reactions necessary for growth and producing bioorganic compounds from CO_2. For example, the energy can be used to convert CO_2 into fatty acids or oils via biosynthetic pathways in the microorganism. Fatty acids are an important class of molecules used to make an array of products including surfactants, solvents, and biodiesel fuels. Other biosynthetic pathways, such as those that produce biopolymers, proteins, or terpene hydrocarbons can also be used.

The source of CO_2 can come directly from an industrial flue gas stream, or from the gasification of agricultural residues (which would also simultaneously provide the H_2 gas component for the bioconversion process). Knallgas microorganisms are able to grow efficiently with established commercial bioreactor technology and bioprocessing equipment, resulting in a readily scalable and deployable system. Plants and algae must be spread out in large flat collection areas, like solar energy installations, to receive sufficient light. In contrast, a knallgas process can scale vertically in compact bioreactors, reducing the footprint and land usage by many orders of magnitude compared to an equivalent algae or plant based process for CO_2 capture and conversion. While this approach differs in several aspects from using plants or algae, it retains the important feature of generating useful biological products from CO_2, including biological oils that can be used in the production of biofuels, chemicals, jet fuel, surfactants, lubricants, solvents, and other industrial products.

Project Process

A Bio-Technological Solution: Microorganisms that Convert CO_2 into Fuels and Chemicals

This project demonstrated processes based on knallgas microorganisms that convert CO_2 to oils or other chemicals that could be used to replace petroleum based products contributing to GHG emissions. Achieving the key technical milestones in this project required a multidisciplinary approach drawing from expertise in chemoautotroph microbiology; gas based bioprocessing, chemical engineering, biomass fractionation, and analytical chemistry. The project was organized as a collaborative effort that included resources from Kiverdi (Berkeley, California), Lawrence-Berkeley National Laboratory (Berkeley, California), Iowa State University (Ames, Iowa), and SRI International (Menlo Park, California).

An exemplary process for converting CO_2 to an oil product includes a bioreactor converting CO_2 to biological oils using a knallgas microorganism, followed by recovery and processing steps similar to those commonly used for isolating similar oil products from other microorganisms such as oleaginous (oily) algae or yeast. To demonstrate this process, experiments were performed during the project with chemoautotrophic knallgas strain *Cupriavidus necator* microorganisms grown on H_2, CO_2, and O_2 gases which represented a simulated mixture of renewable H_2 with waste CO_2. The project included optimizing lab-scale growth of biomass and production of oils, scale-up and testing the lab-scale process, and design of a mobile pilot scale (50 Liter) bioreactor system for knallgas microbes.

Strains were grown in high-throughput small scale systems (e.g. 160 ml gas-tight serum bottles) to evaluate numerous strain candidates, and in larger scale bioreactors to produce biomass and oil product samples from CO_2. Both batch and continuous bioreactor runs were performed. Development, optimization, and scale-up were performed on the gas culturing, cell harvest, lipid extraction, and oil purification steps of the overall process for converting CO_2 to biological oils. The majority of strains tested were grown at standard bioprocess temperatures (e.g. 25-35 °C). One thermophilic knallgas strain was grown on H_2 and CO_2 at 75°C.

Project Results

The project established methods and protocols for growing knallgas microorganisms using H_2 and CO_2 both at laboratory scale and 20 L pilot scale in stirred tank bioreactors. The project's results show that, in addition to H_2, CO_2, and O_2 gaseous substrates, the only additional requirements for growth of the knallgas microorganisms were minor inorganic mineral nutrients (e.g. nitrogen (N), phosphate (P), and potassium (K) sources), similar to those provided by fertilizers used to grow plants and agricultural crops.

Using results from lab-scale optimization, growth of *C. necator* was successfully scaled to 20 liter bioreactors, with total cell densities reaching 22 grams per liter. The project culminated with design of a mobile 50 liter bioreactor pilot plant to test at potential production sites.

This project demonstrated that knallgas microorganisms can be used to convert waste CO_2 to biochemical products using conventional bioprocessing technology. Furthermore, in previous work, metabolic engineering experiments with the knallgas strain *C. necator* resulted in production of alternative high energy density and high value hydrocarbons. This process demonstrates the applicability of the processes to a broad range of potential products that could be accessed through modern strain engineering technologies.

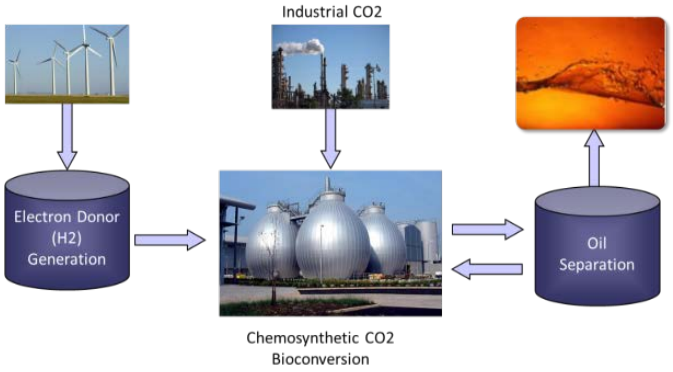
Biological Conversion of CO_2 Using Chemoautotrophic Knallgas Microorganisms

Kiverdi is developing an alternative approach to renewable products from CO_2 utilizing chemoautotrophs (Figure 1). The core of the process relies on well-established, scientifically characterized microorganisms that produce fatty acids and other biological oils and hydrocarbons. The microbes use (1) CO_2 as a carbon source and (2) hydrogen (H_2) as a source of reducing electrons. Thus, the microorganisms are hydrogenotrophic (H_2-oxidizing), and chemoautotrophic (CO_2-fixing) microbes that act as biological catalysts for the chemically challenging steps of binding together carbon atoms from CO_2 to form multi-carbon organic molecules, including oils and hydrocarbons. The steps in the carbon fixation reaction require energy and reducing electrons, which are provided by H_2 and, in some cases CO. Kiverdi's approach exploits the unique properties of a certain class of hydrogenotrophic microorganisms able to produce high-energy-density chemicals and fuels from H_2 and CO_2, known as knallgas microorganisms. Knallgas microbes are a phenotypical group that can grow on CO_2 using a mixture of H_2 and O_2 (knallgas) to provide intracellular energy for CO_2-fixation; synthesis of biomolecules; and cellular growth and maintenance.

Using chemoautotrophs, such as knallgas microorganisms, for carbon fixation, instead of photoautotrophs such as algae, enables use of well-known bioprocessing technology including

conventional microbial culturing equipment and designs; proven in many industrial processes. Bioreactor equipment may be used in the process that is similar in many respects to that used in common fermentations to make beer or wine. Similar bioreactor designs and equipment to that widely used in waste-water treatment may also be applied as well.

Figure 1: Technological Concept



Project Benefits

This project advanced the process of capturing and converting CO_2 to chemical products with knallgas microorganisms to possibly reduce California's dependence on petroleum. The project demonstrated that knallgas biological conversion technology can be developed and utilized as a platform to provide a diversity of potential products, including fuels, oils, and other industrial chemicals. Furthermore, the project demonstrated that the process be can readily scaled and deployed with existing bioreactor and bioprocessing technology.

Some advantages of the approach are:

- Feedstock flexibility to use CO_2 and other carbon-based wastes that are abundant and low cost.
- Does not compete with food production, nor cause major disruption in land use or natural habitats, nor consume large quantities of fresh water or fertilizer.
- Versatility of products, allowing high value or special purpose chemicals to be targeted.

Technology developed through this project can help meet the intent of California's energy policy regulations, including the Global Warming Solutions Act (AB 32), the Renewable Portfolio Standard, and the Low Carbon Fuel Standard (LCFS) by significantly redirecting released CO_2 from industrial sources such as fossil fuel power plants, cement manufacture, oil refining, steel production, ethanol fermentation, and geothermal power plants into the production of commercial chemical and fuel products.

Producing oils and other chemicals from CO_2 can also create jobs in California and meet future customer needs for drop-in vegetable oils and animal fats and oils (oleochemical) products at a price uncoupled from petroleum or volatile commodity oil-crop prices. Kiverdi is actively

working to further improve these novel technologies through a multi-disciplinary approach drawing on many different fields of expertise to advance the technology to commercialization on a wide scale.

CHAPTER 1:
Introduction

1.1 Knallgas Conversion Technology

1.1.1 Range of Feedstocks

A key advantage of the technology is the use of gaseous intermediates in the process, a mixture of CO_2, H_2, and sometimes CO, which can be generated from a wide range of low-cost waste feedstocks using established, proven methods. When CO is present in the gas mixture along with H_2 and CO_2, the gas mixture is generally called a synthesis gas or syngas. In principal, any carbon-based, combustible gas, liquid, or solid can be used to produce H_2 by the established processes of gasification or steam reforming. Examples of the wide range of carbon-based feedstocks that can be used to produce H_2 by gasification or steam reforming include:

- natural gas
    - conventional
    - stranded or flared natural gas
- municipal solid waste (MSW)
- biogas
- biomass
    - agricultural residues
    - forestry residues
    - wood waste
        - e.g. collected in hazardous fuel reduction treatments for forest fire prevention or generated at lumber mills, paper mills, and woodshops
    - lignin
        - e.g. residual material from cellulosic ethanol or pulping process at paper mills
- various industrial plant tail gases
- landfill gas
- pet coke (low value waste product from oil refining)
- liquid petroleum gas (LPG) (co-product of natural gas production)

The gasification or steam reforming of a carbon-based feedstock such those given above will simultaneously produce the CO_2 and/or CO that can be used as a carbon source in the bioprocess. Hence by generating both H_2 and CO_2 and CO, gasification and steam reforming

simultaneously generate both the energy source and carbon source needed in a knallgas bioprocess.

Alternatively, any CO_2 emission-free method of electricity generation, including solar, wind, hydroelectric, geothermal, and nuclear power, can be used to produce H_2 from water by the established method of electrolysis. This renewable or low-carbon H_2 thus generated, can then be used to capture and convert CO_2 emitted from point sources such as industrial flues. The CO_2 serves directly as the carbon source for growth. An input gas stream is fed into the growth reactor, where the CO_2 dissolves in the media and the chemoautotrophic microbes' uptake and fix the CO_2 directly. The microbes then convert the assimilated CO_2 into lipids, hydrocarbons, or other high-value products using electrons and hydrogen atoms from H_2.

1.1.2 Range of Products

The ultimate goal is a technological platform for converting various low cost, abundant feedstocks—ranging from CO_2 point source emissions, to waste or purpose grown biomass—into medium- and long- carbon-chain oleochemicals. These oleochemical molecules have many uses, ranging from surfactants and consumer packaged goods to feedstocks for drop-in diesel and jet fuel replacements. Particular classes of oleochemicals being targeted for production from CO_2 are oleochemicals derived from mid- to long- carbon-chain fatty acids. Mid- to long-carbon-chain fatty acid products are inaccessible using current syngas conversion technologies, including chemical methods such as Fischer-Tropsch (F-T), and biological processes such as gas fermentation using *Clostridium* strains.

Biological methods for conversion of CO_2 or syngas using *Clostridium* have been the focus of significant public and private investment. However, this work has focused on producing short-chain carbon molecules such as the C2 compounds acetic acid and ethanol. Anaerobic respiration, which is central to the production of these C2 compounds by *Clostridium*, uses CO_2 as part of core metabolism. Although using an anaerobic respiratory pathway, results in high product yields, it is inherently limited to smaller carbon chain products, typically C1 through C4 products (e.g., CH_4, CH_3COOH, CH_3CH_2OH, or $CH_3(CH_2)_2COOH$). The phenotypic types of strains used to produce such C1-C4 products from CO_2 through anaerobic respiratory pathways are called methanogens or acetogens.

The larger molecules targeted by the proposed knallgas conversion technology are typically more valuable and often have higher energy densities when used as fuel. There are many other desirable characteristics of product molecules having longer carbon-chain lengths. The proposed technological platform creates the opportunity to fundamentally and disruptively advance the versatility, efficiency, and practicality of waste carbon conversion processes by extending both the range of products for any particular feedstock, and the range of feedstocks for any particular oleochemical product.

1.1.3 Alternate Approaches to CO_2 Conversion

Chemical methods for the conversion of CO and CO_2 into organic molecules exist as well, the best known of which is the Fischer-Tropsch (F-T) process. This process has been used to produce longer carbon chain length molecules chemically from syngas, yielding middle

distillates such as jet fuel and diesel (C8-C18), as well as much longer chain lengths extending out to heavy waxes (C30+). Like many chemical processes, F-T requires harsh reaction conditions and large capital outlays.

The question is, can longer carbon chain length molecules be produced efficiently from syngas or H_2/CO_2 gas mixes in a bioprocess?

One option that has been explored in the biological gas-to-chemicals space is to simply post-process chemically the short carbon chain products of the anaerobic syngas fermentation (e.g. C2) into desired longer carbon chain products. For example, ethanol can be chemically dehydrated to ethylene under elevated temperatures. The polymerization of ethylene to produce longer molecules is a standard commercial chemical process used to produce both polyethylene and fatty alcohols. However, it is generally simpler, more efficient, and less expensive to avoid major post-processing of products such as this, if possible.

Another option would be to metabolically engineer anaerobic respiratory pathways using CO_2 electron acceptor in acetogens or methanogens, which naturally produce C1-C4 molecules, to produce longer carbon chain products. While an enticing prospect in the abstract, this level of metabolic engineering is well beyond current precedent.

A third option would be bioprospecting for a microorganism that naturally converts CO_2 through an anaerobic respiratory pathway into longer-chain products (e.g. C5+). As discussed in the next section, there is a very wide diversity of hydrogenotrophic microorganisms, many of which haven't been studied or characterized. However promising in the long run, this would also be a very uncertain endeavor in the short run, and is well beyond the scope and resources of this project. Such microorganisms, if they exist at all, would likely be hyperextremophiles/hyperbaryophiles occupying inaccessible environments such as deep under the earth's surface.[1]

Instead, Kiverdi has pursued a practical, lower-risk compromise that offers the potential for a breakthrough with the near-term likelihood for success in the R&D effort. This approach is to develop a gas bioprocess targeting products synthesized through naturally-occurring and well-known anabolic biosynthesis pathways, such as the fatty acid and lipid (TAG) biosynthetic pathways discussed below. This is similar in practice to the incumbent gas bioprocesses using acetogenic and *Clostridium* bacteria, which target ethanol and other short-chain products through naturally occurring and well-known anaerobic respiratory pathways. The similarity in general approach lies in the development of gas bioprocesses that target products through naturally occurring and well-known metabolic pathways, as opposed to attempting extreme synthetic biology or highly uncertain bioprospecting.

1.2 Chemistry and Biology of Knallgas Bioconversion

Much of the CO_2 on Earth is captured and fixed via non-photosynthetic pathways. This CO_2 is reduced by chemoautotrophs utilizing diverse reducing agents ranging from H_2, to various metal ions, and sulfides. To date, chemoautotrophs have not been extensively developed for

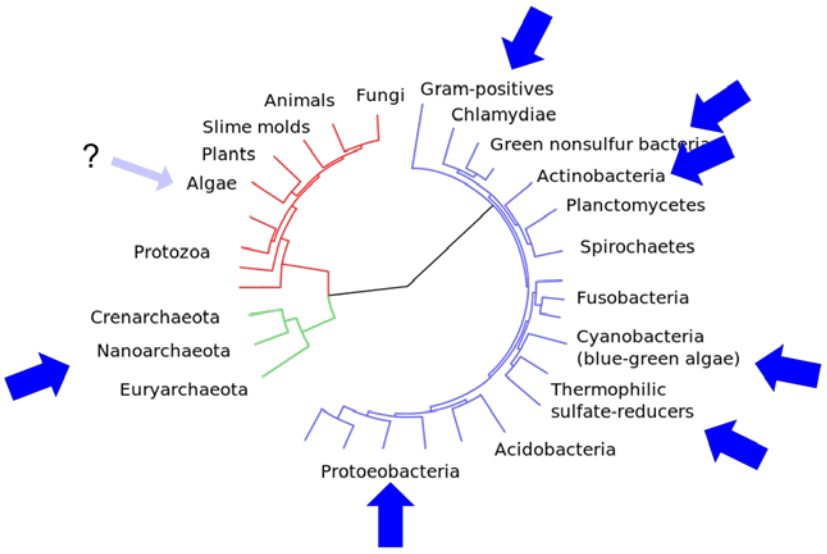
biological production of organic molecules including fuels. However, recent work with broad-host genetic methods has shown them to be genetically manipulable.

1.2.1 Knallgas Strains

In this class of chemoautotrophs (hydrogenotrophs) the energetic force for the reaction, the electron donor, comes from H_2. These organisms are targeted because there are many ways to efficiently generate H_2 from renewable, CO_2 emission-free energy sources, such as solar, wind, and hydroelectric power.[2] H_2 can also be generated from the gasification of biomass or the steam reforming of methane.

The use of an H_2 electron donor in chemoautotrophy - called hydrogenotrophy - is found across the microbial biosphere and is likely an ancient trait dating back to when the earth had a reducing atmosphere (i.e. without free O_2). Some species exhibiting this trait are among the most ancient members of the bacterial domain. Most hydrogenotrophic strains are poorly or not at all characterized, owing to the extreme environmental conditions under which they grow, which are difficult to reproduce in the lab. Several bacterial and archaeal phyla contain hydrogenotrophic microbes, as indicated in Figure 2.

Figure 2: Diverse Branches of Microscopic Life Exhibiting Hydrogenotrophy



Blue arrows indicate the diverse branches of life that contain members exhibiting hydrogenotrophy (ability to grow on H_2)

1.2.2 Central Metabolism Chemistry

The generic chemoautotrophic reaction is given by:

$$\text{Electron Donor} + CO_2 + H_2O + \text{Electron Acceptor} \rightarrow \text{Cell mass} + \text{Chemical co-products}$$

The oxidation of the electron donor by the electron acceptor in respiration provides the chemical energy needed for the fixation of CO_2 into biomolecules. The electron donor is also used to reduce the carbon on CO_2. The biomolecules produced from CO_2 compose the cell mass. These biomolecules constituting the cell mass include proteins, polysaccharides, nucleic acids - and most importantly in the current context - lipids, oils, and fats. The chemical co-products are generally the waste products of the chemoautotrophic metabolism that diffuse into the extracellular environment; although in some cases these waste products can also include organic biomolecules of value (e.g. ethanol, methane, acetic acid etc.). No fixed carbon nutrients or light are required for growth and synthesis of biomolecules.

In the subcategory of chemoautotrophs known as hydrogenotrophs, the "Electron Donor" is H_2. Some hydrogenotrophs are also carboxydotrophs, meaning CO-oxidizing. In the case of these organisms, CO can also play the role of electron donor. In the particular subcategory of hydrogenotrophs known as knallgas microorganisms, the "Electron Acceptor" is O_2. For knallgas microorganisms the only chemical co-product is water. In other words, all of the carbon in the CO_2 reactant goes into the cell mass which is composed primarily of oils, fats, proteins, polysaccharides, and other biopolymers. This stands in marked contrast to the anaerobic acetogens and Clostridia used in incumbent gas fermentations where the majority of the carbon in CO_2 reactant goes into chemical co-products such as acetic acid, ethanol, etc., instead of cell mass.

In knallgas microorganisms, and hydrogenotrophs generally, hydrogenase enzymes convert H_2 into intracellular reducing equivalents such as nicotinamide adenine dinucleotide hydride (NADH), which in turn are used for biosynthesis and respiration. The reducing electrons donated from H_2 are utilized for the reduction of NAD^+ (and/or other intracellular reducing equivalents) according to the following reaction:

$$H_2 + NAD^+ \rightarrow NADH + H^+$$

NADH functions as an intracellular H_2 carrier that solubilizes and keeps contained within the cell the otherwise poorly soluble H_2 molecule. Some knallgas microbes additionally contain carbon monoxide dehydrogenase (CODH), an enzyme that performs the water-gas shift reaction, converting CO and water to CO_2 and H_2:

$$CO + H_2O \rightarrow H_2 + CO_2$$

Alternatively, some versions of CODH catalyze the reduction of NAD^+ to NADH directly:

$$CO + H_2O + NAD^+ \rightarrow CO_2 + NADH + H^+$$

The H_2 (and/or NADH) and CO_2 thus formed from CO through this enzymatically-catalyzed reaction, can then proceed into the hydrogenotrophic metabolism of the knallgas microorganism. Knallgas strains with both hydrogenase and CODH enzymes are able to use both the H_2 and CO content of syngas directly.

The ubiquitous intracellular energy carrier, adenosine triphosphate (ATP), which is required to carry out CO_2 capture and fixation, is produced through the oxyhydrogen/knallgas reaction:

$$2H_2 + O_2 \rightarrow 2H_2O + \text{energy}$$

This highly energetic reaction is enzymatically linked to the production of ATP through oxidative phosphorylation.[3] The oxidation of hydrogen can act as a proxy for the light reaction in photosynthesis in generating both reducing equivalents (e.g. NADH or NADPH) and ATP. Therefore, in place of the following light dependent photosynthetic reaction:

$$2H_2O + 2NADP+ + 2ADP + 2P_i + \text{light} \rightarrow 2NADPH + 2H^+ + 2ATP + O_2$$

a net oxyhydrogen reaction such as the following can occur:

$$\tfrac{1}{2}O_2 + 2NADP^+ + 2ADP + 2P_i + 3H_2 \rightarrow 2NADPH + 2H^+ + 2ATP + H_2O$$

in dark conditions (i.e., in the complete absence of visible electromagnetic radiation), with H_2 and O_2 acting in the place of photons for the production of intracellular reducing equivalents (e.g. NADPH) and ATP needed for biosynthesis.

Table 1 summarizes important pathways or enzymatic steps that are present in a knallgas strain useful for converting H_2/CO_2 gas mixes, or syngas (i.e. $H_2/CO/CO_2$ gas mixes), into fatty acids such as C16 palmitic acid.

Table 1: Important Pathways or Enzymatic Steps Present in Knallgas Strains

Enzyme or Pathway	Formula
Hydrogenase	$H_2 + NAD^+ \rightarrow NADH + H^+$
Carbon Monoxide Dehydrogenase	$CO + H_2O \rightarrow H_2 + CO_2$
Oxidative Phosphorylation	$H_2 + 1/2O_2 + 2\text{-}3ADP + 2\text{-}3P \rightarrow H_2O + 2\text{-}3ATP^*$
Calvin Cycle or Reverse TCA	$2CO_2 \ldots \rightarrow \ldots \rightarrow \ldots \rightarrow$ Acetyl-CoA
Fatty Acid Biosynthesis (sample)	8 Acetyl-CoA + 7ATP + H_2O + 14NADH + $14H^+ \rightarrow$ Palmitic acid + 8CoA + $14NAD^+$ + 7ADP + 7P

Importantly, all knallgas strains have a carbon-fixing pathway, such as the Calvin cycle or the reverse tricarboxylic acid cycle (rTCA - also known as the reverse citric acid cycle or reverse Krebs cycle), which converts CO_2 to the central metabolic intermediate acetyl-CoA. Acetyl-CoA is the entry molecule for the fatty acid biosynthesis pathway, as described below.

1.2.3 Fatty Acids, Lipids, and Other Products

The use of anabolic biosynthetic pathways for product synthesis in a gas bioprocess (H_2/CO_2 or syngas inputs) is a key differentiator for the proposed platform. Anabolic pathways generate the basic building blocks of life. These pathways are used for the synthesis of the complex chemicals and materials comprising living cell mass or tissue in all multicellular life including higher order plants, as well as in the known mesophilic microorganisms found in surface

environments on earth. Generally, anabolic pathways consume some intracellular carrier of free energy generated through respiration such as ATP. Often anabolic pathways also consume reducing equivalents such as NADH or NADPH as well.

The particular molecules of interest, fatty acids, are produced through an anabolic pathway called fatty acid biosynthesis (Figure 3). Starting with the central metabolic intermediate acetyl-CoA, which has two carbons, the reaction in the fatty acid biosynthesis pathway proceeds to join two carbon acetyl-CoA units together so as to produce a longer carbon chain fatty acid. As an example, the C16 fatty acid - palmitic acid (Figure 4), is formed through fatty acid biosynthesis according to the following net reaction formula:

8 Acetyl-CoA + 7ATP + H_2O + 14NADPH + $14H^+$ -> Palmitic acid + 8CoA + $14NADP^+$ + 7ADP + $7P_i$

As discussed previously, all knallgas microorganisms have the ability to produce from CO_2 the central metabolic intermediate acetyl-CoA, which in turn enters into fatty acid biosynthesis.

Figure 3: The Fatty Acid Biosynthesis Pathway Starting from Acetyl-CoA

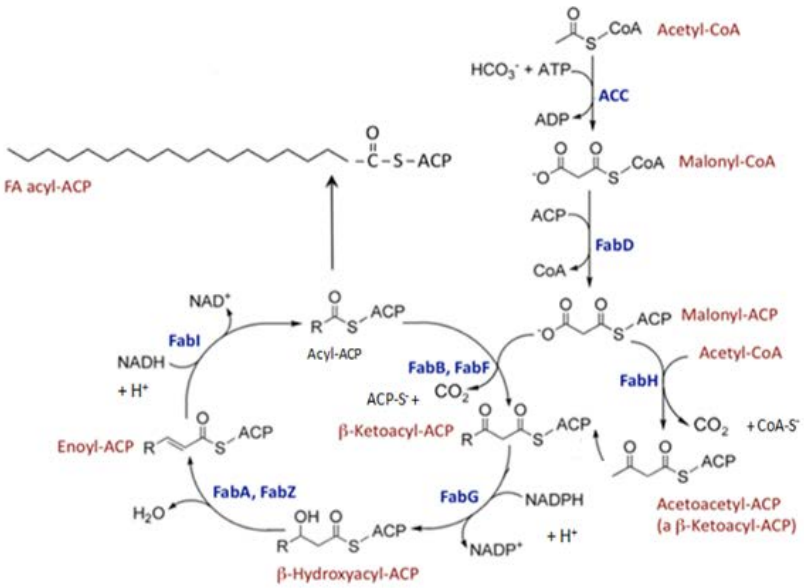
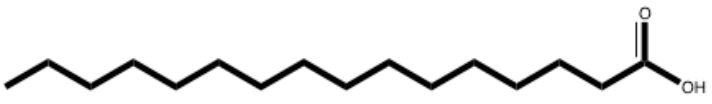
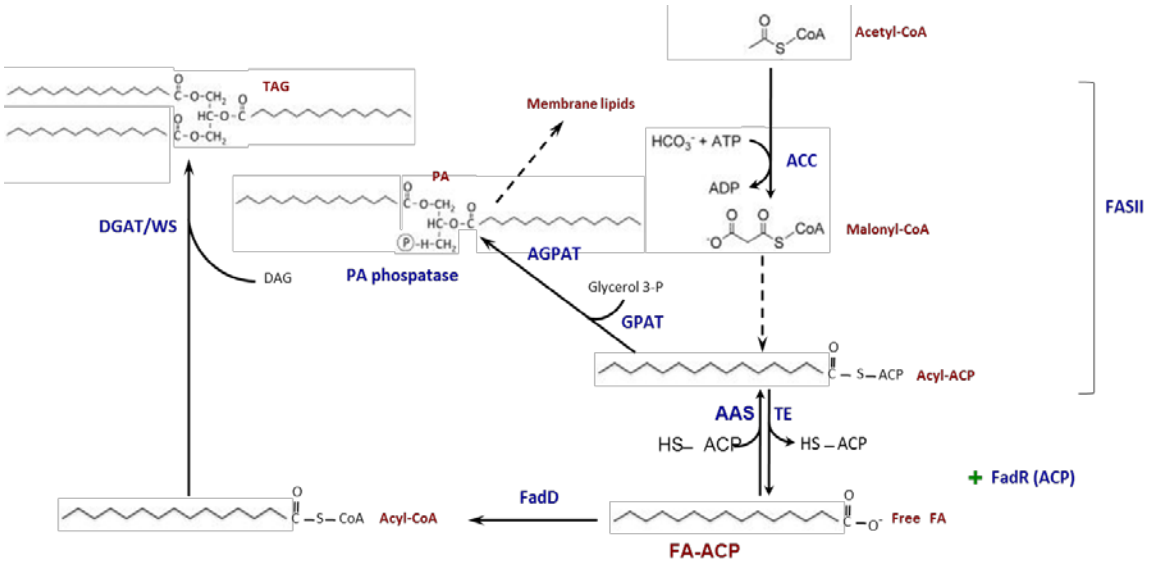


Figure 4: Molecular Structure of C16 Palmitic Acid



Additional enzymatic steps then convert the fatty acid compounds into biological oils in the form of triacylglycerides (TAGs) or as structural membrane lipids (Figure 5).

Figure 5: Synthesis of Triacylglycerol Oils (TAGs) and Membrane Lipids from Fatty Acids



Source Rock, C. O. and S. Jackowski (2002). "Forty Years of Bacterial Fatty Acid Synthesis." Biochemical and Biophysical Research Communications 292(5): 1155-1166.; and Dr. Christer Jansson, Lawrence Berkeley National Laboratory (LBNL), private communication, 2011.

Other examples of well-known and characterized anabolic pathways include amino acid biosynthetic pathways, isoprenoid or terpenoid biosynthetic pathways, and polysaccharide pathways. All plant and animal derived oils, fats, and hydrocarbons are produced through either the fatty acid biosynthesis pathway or an isoprenoid pathway. Some types of plant and animal derived fatty acids, their traditional sources, and commercial uses, are listed in Table 2.

Table 2: Traditional Natural Sources of Various Fatty Acids and Major Applications

C-Chain Length	Major Markets
C_{20}-C_{24} (Peanut & Tree Oils)	Lubricants, Conditioners, Surface Modification
C_{16}-C_{18} (Palm Oil)	Laundry Detergents, Specialty Surfactants, Pharmaceuticals
C_{14} (Sperm Whale Oil)	Cosmetics, Skin Care/Emollients, Candles
C_{12} (Coconut Oil, Palm Kernel Oil)	Most broadly used fatty acid – Soaps, Light Duty Cleaners, Cosmetics

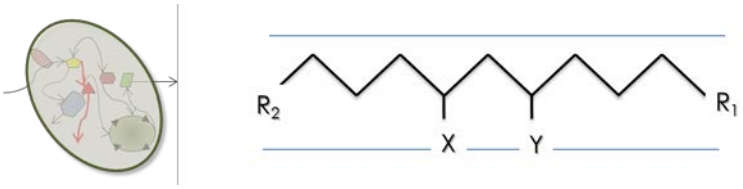
Biological oils and hydrocarbons produced through isoprenoid pathways include citrus oil and orange oil (primarily composed of limonene), shark liver oil (squalene), and turpentine (from pine trees; mainly the monoterpenes alpha-pinene and beta-pinene with lesser amounts of carene, camphene, dipentene, and terpinolene). Some of the traditional plant and animal

sources of fatty acids and terpenes, while being natural non-fossil sources, are nonetheless extremely environmentally and ecologically problematic (e.g. palm oil, palm kernel oil, sperm whale oil, and shark liver oil).

Both the fatty acid biosynthesis pathway and isoprenoid pathways, occur naturally in knallgas microorganisms. Hence, in principle, knallgas microorganisms should be applicable to the production from CO_2 of any oil, fat, or hydrocarbon known to be produced by plants or animals. In practice, depending on the exact molecular structure targeted, a relatively small number of enzymatic steps may need to be genetically engineered into the microorganism. However, the general metabolic framework for producing these biological oils and fats from CO_2, such as those listed, and many more, are already naturally present in knallgas microorganisms. Thus the potential of an approach leveraging anabolic pathways in knallgas microorganisms is enormous.

The mid- to long- carbon chain organic molecules being targeted are most directly synthesized through either the fatty acid biosynthetic or an isoprenoid pathway. R1, R2, X, and Y represent possible functional groups that can be attached to a carbon-chain backbone such as COOH, OH, H, and aliphatic or aromatic side groups (Figure 6). Kiverdi has developed expertise in the enzymatic modification and optimization of both of the fatty acid and isoprenoid pathways in a knallgas microorganism as will be discussed in subsequent sections. Generally speaking, the fatty acid biosynthesis pathway is better suited for the production of straight chain, saturated molecules, and the isoprenoids pathways are better suited for the production of unsaturated, branched, or cyclic molecules. While the molecules produced through the fatty acid and isoprenoid pathways tend to have high energy density, and hence potential applicability as transportation fuels, they often have current applications with higher value than as a general transportation fuel. Such higher per unit value applications can be as a specialty fuel-type or fuel-additive, or as a finished or intermediate organic chemical. For example some of the higher value organic chemical applications of various fatty acids listed in the preious table.

Figure 6: General Molecular Structures Targeted for Synthesis from CO_2



The possibility of targeting cyclic and aromatic products through anabolic pathways from CO_2 or syngas feedstock represents a particularly unique niche in the gas-to-chemicals space. Currently there are no commercial direct gas-to-aromatics or gas-to-cyclics conversions. For example, producing aromatics or cyclics through F-T requires additional post-process cracking and isomerization; and through the methanol-to-gasoline process (MTG),[4] it is necessarily a two-step process (i.e., syngas-to-methanol and then methanol-to-aromatics and cyclics) with relatively low product specificity in the MTG step. Biological conversion could enable the

synthesis from gaseous energy and carbon feedstocks of plastic monomers or gasoline octane boosters, which often have aromatic or cyclic molecular structures, through isoprenoid and aromatic amino acid pathways.

1.2.4 Strain Selection and Metabolic Engineering Strategy

Using knallgas microorganisms, and the highly energetic knallgas reaction in respiration, enables the efficient production of ATP from gaseous feedstocks (e.g. H2/CO2 or syngas mixtures). The efficient production of ATP in turn is necessary for efficient synthesis of molecules through anabolic pathways such as the fatty acid and isoprenoid pathways. However, while the efficient production of ATP, is necessary for an efficient oil or lipid production process, it is not sufficient by itself to ensure an efficient bioprocess for the production of oils. A microbe could efficiently produce ATP, but then expend the ATP in the biosynthesis of other biochemicals than those targeted, such as proteins or polyhydroxyalkanoates (PHAs). For example a knallgas microorganism known to accumulate high amounts of polyhydroxybutyrate (PHBs), *Cupriavidus necator* (also known as *Ralstonia eutropha*), directs the bulk of its ATP, reducing equivalents, and carbon towards PHB synthesis, rather than fatty acid synthesis.

There are four factors that need to be fulfilled for high efficiency production of oils from CO_2 through the fatty acid biosynthesis pathway:

- High efficiency (i.e. low ATP requirement) in the CO_2-fixing pathway or cycle,
- High efficiency in producing ATP through respiration (i.e. high amounts of ATP per H_2 consumed in respiration),
- High flux of carbon down FA biosynthesis pathway (as opposed to pathways leading to other types of molecules), and
- High efficiency of fatty acid biosynthesis (less ATP leads to increased efficiency).

A similar set of criteria apply for isoprenoid pathways, substituting the isoprenoid pathway for FA biosynthesis.

The choice of knallgas microorganisms for the efficient production of ATP in respiration follows naturally from thermodynamic considerations. The highly energetic knallgas reaction means that a large amount of energy in the form of ATP can be recovered for each H_2 directed into respiration. However, the ability of a given knallgas microorganism to direct a high flux of carbon down the FA biosynthesis pathway, thereby enabling the accumulation of a high percentage by weight of lipid, is not a given for all knallgas microorganisms. Therefore, in this project, an empirical approach was used to identify candidate knallgas strains that exhibit an ability to accumulate rich cellular lipid content. The high biomass lipid content reflects the proportion of carbon, reducing equivalents, and ATP directed towards lipid synthesis, as opposed to other biosynthetic pathways in the microorganism. Microorganisms were identified with the knallgas phenotypes that are also naturally high oil producers under certain conditions. Putative genera containing knallgas strains were identified, that have strains reported to be able to store lipid content, in some cases, over 70% of biomass by dry weight.[5]

This phenotype reflects a natural ability to direct a high carbon flux down the FA biosynthetic pathway, and to direct reducing equivalents towards the synthesis of lipid products, rather than other biomass components. The ability to store high lipid content is a key attribute in ensuring the overall efficiency of the proposed lipid production process.

Additional attributes that can strongly affect the performance of a bioprocess were evaluated during knallgas phenotype selection, including the biomass titers that could be reached, the doubling time of the cells under exponential growth conditions, and the resistance of the culture to contamination. In these respects and many others, promising knallgas candidate strains were identified including strains that were reportedly grown to dry cell densities of over 90g/liter on gaseous energy and carbon source,[6] and strains with reported doubling times down to one hour.[7] These strains also showed resistance to contamination reflected by almost 300 days of continuous growth in a chemostat without process shut down.[8] Long continuous runs without any shutdowns indicate robustness against contaminants. The chemoautotrophic doubling times found for knallgas microorganisms also tend to be substantially shorter than those observed with anaerobic acetogens and methanogens, which suggests a connection between the superior energy yield of respiration and faster growth rates as well. Also promising, is that the environments that many candidate knallgas strains have evolved in and have been isolated from naturally contain many of the impurities found in syngas and industrial flue gases (e.g. tars, sulfides, various dissolved metal ions). It is thus likely that strains can be identified that are more robust and tolerant to such impurities than strains used in the incumbent syngas fermentations.

In summary, the following main points motivated the choice to apply 1) anabolic metabolic pathways and 2) knallgas microorganisms to the production of higher value organic molecules from CO_2 in a gas bioprocess:

- Hydrogenases convert H_2 into reducing equivalents for biosynthesis and respiration.
- In some strains, CODH performs the water gas shift converting CO to CO_2 and H_2.
- Knallgas microbes produce far more ATP per H_2 consumed in respiration than microbes used in incumbent gas fermentation processes:
    - At least 8x more ATP per H_2 consumed than methanogenic or acetogenic hydrogenotrophic microorganisms.
- Anabolic synthesis is more efficient because of higher ATP yield, and more carbon can be directed into the desired biomass product, rather the respiratory products such as methane or short chain organic acids (C2-C4).
- Higher energetic yield allows for products beyond short-chain alcohols.
- Expanded metabolic versatility.
- Possibility of fuel molecules with higher energy densities and greater infrastructure compatibility including drop-in diesel and jet fuel.

- Ability to synthesize oil more efficiently than incumbent microorganisms.

In order to lay the groundwork for the proposed gas bioprocess platform based on anabolic pathways, and for implementing particular pathways leading to high energy density molecules such as fatty acid biosynthesis and isoprenoid pathways, the main technical objectives in this project pursued optimizing and scaling chemoautotrophic cultivation conditions using H_2 electron donor and CO_2 carbon source for efficient biosynthesis and production of biomass. Establishing the optimized and scaled-up cultivation of knallgas microorganisms in turn lays the groundwork for applying techniques, processes, and tools for genetic manipulation of knallgas platform microbes having the desired metabolic capabilities; to target production of desired fuel and chemical molecules from CO_2 carbon input.

Success in the goals and objectives of this project can lead to a bioprocess with improved flexibility and scalability over incumbent approaches to biofuel and biochemical production. This flexibility and scalability is enabled by using H_2/CO_2 gas mixes, or syngas, or $H_2/CO/CO_2$ gas mixes generally. In particular, obtaining the goals and objectives would facilitate the targeting of higher carbon number products, and more drop-in molecules, in a CO_2 or syngas conversion bioprocess, than can be realized using incumbent syngas conversion bioprocesses.

1.3 Other Considerations

1.3.1 Comparison to Other Biological Approaches

One of the most commonly suggested paths for capturing and using CO_2 is through photosynthesis pathways occurring in plants and algae. The possibility of applying fast-growing algae to CO_2 capture is a tantalizing idea that has received considerable attention over many decades. In this approach, generally CO_2 is fed into open ponds or photobioreactors that contain the algae, which in the presence of light assimilate CO_2 into carbohydrates and oils. When the growth of the algae is complete, the algae is dried and processed to extract useful materials such as the accumulated oils. Among the difficulties with the algae approach are the large volumes of water required as well as land that must be utilized, susceptibility to contaminants and inclement weather conditions in open cultivation systems, and sensitivity to light conditions and climate that constrain and greatly limit viable geographical locations.

The knallgas approach achieves similar product profiles without many of the disadvantages of photosynthetic algal production. The same types of biological oils used to produce biofuels and oleochemicals that are produced from CO_2 by algae, can also be produced with knallgas microorganisms. Kiverdi has identified knallgas microbes that can accumulate lipids to over 50% of cell dry weight, similar to the high content of certain strains of microalgae. Just as algae oil can be converted into biodiesel or hydrotreated to produce drop-in diesel or Hydrotreated Renewable Jet (HRJ) fuel, so to can the oils from these chemoautotrophic knallgas microbes.

As discussed, there are more incumbent gas bioprocesses under development that apply hydrogenotrophic acetogens and Clostridial microorganisms. Such bioprocesses are being developed by companies including Ineos Bio, Lanzatech, and Coskata. The microorganisms used by these companies are strict anaerobes. In contrast, knallgas microorganisms tolerate the presence of O_2, and can use it in respiration. In this and other ways, knallgas microorganisms

are substantially different than the hydrogenotrophic acetogens and Clostridial microorganisms used in incumbent gas bioprocesses. The use of knallgas bacteria allows one to practically target higher molecular weight, longer carbon-chain, and higher carbon-number products in a gas bioprocess, than what would be practical with these incumbent gas bioprocesses. Such molecular targets enabled by the use of knallgas microorganisms include fatty acids, TAGs, and terpenes. These higher carbon number molecules have far broader application and value in the organic chemicals market than the low carbon number molecules produced through incumbent gas bioprocesses, and notably can be used to produce drop-in substitutes for diesel and jet fuel.

The application of knallgas microorganisms in engineered bioprocesses has received relatively scant attention, particularly compared to photoautotrophs or sugar-consuming heterotrophs. However, the application of knallgas microorganisms to CO_2 capture and conversion is not entirely unprecedented. Knallgas bioprocesses showed great promise in Apollo-era NASA research on CO_2 uptake for closed-loop life support and in-situ resource utilization systems. Such systems were intended to regenerate the air and recycle carbon in spaceship cabins on long-duration, deep-space missions without resupply;[9] and may become relevant in that context again as research to support a mission to Mars progresses. It is hoped these types of hydrogenotrophic microorganisms can also be applied to tap CO_2 and other low cost wastes, along with clean, abundant renewable feedstocks, for the industrial scale production of organic chemicals, materials, and liquid fuels from here on Earth.

1.3.2 Comparison to Other Chemical Approaches

Converting CO_2 to methanol using renewable H_2 through the methanol synthesis process is another approach that has been proposed for the beneficial use of CO_2.[10] Methanol synthesis is a commercial, high temperature and high pressure catalytic gas-to-liquid (GTL) reaction. Methanol can be used as a liquid fuel, however its fuel characteristics are relatively poor including lower energy density and higher corrosivity than conventional petrofuels such as gasoline or diesel. The option exists to subsequently convert methanol to a higher value drop-in fuel through the methanol-to-gasoline (MTG) process. However the MTG process has only had marginal commercial viability to date. Currently methanol is generally synthesized from synthesis gas (syngas) produced from natural gas through the methane steam reforming reaction (MSR). Natural gas is a non-renewable, fossil fuel; so ultimately, when commercially produced methanol degrades, decomposes, or is combusted at the end of its lifecycle, fossil carbon is released to the atmosphere as CO_2. It is unlikely that the low value (i.e. per unit price; $/kg or $/gal) of methanol could justify the use of renewable H_2 generated from non-fossil sources, which is currently relatively expensive compared to H_2 or syngas from MSR of natural gas. Given these combination of factors, it seems likely methanol will continue to be produced through the current commercial pathway from natural gas feedstock, rather than from waste CO_2 and renewable H_2, for the foreseeable future.

The Fischer-Tropsch (F-T) thermochemical process is another high temperature and high pressure catalytic GTL reaction. In contrast to methanol synthesis, F-T can produce middle distillate, liquid hydrocarbons, directly. F-T provides a means of producing high energy density liquid fuels such as diesel and jet fuels that have great compatibility with the current

transportation infrastructure and fleet. However the F-T process has only seen limited commercial application to date. Wider application of F-T is mainly hinder by two factors: 1) high capital costs and 2) relatively low per unit value products.

F-T tends to produce relatively lower value, linear carbon-chain hydrocarbons. Unlike the MTG reaction discussed previously, the F-T reaction is not efficient for producing ringed, branched, or aromatic carbon backbones, which tend to command a higher price on a per unit basis (e.g. $/gal or $/kg). In order to produce such cyclic, aromatic, and branched carbon-back bone structures through F-T, additional post-process isomerization steps would be required. The F-T reaction is also not an efficient path to producing slightly oxygenated molecules that tend to command a higher price as an intermediate or finished chemical, than the totally deoxygenated linear hydrocarbons produced by F-T. The oils and fats produced by biological organisms such as algae, plants, animals, and knallgas microbes are often slightly oxygenated. For example, fatty acids, which comprise a long acyl chain terminated with a carboxylic acid (figure 1.4), are a common constituent of biological oils and fats. Such fatty acid molecules generally command a higher price per unit weight than linear, deoxygenated hydrocarbons, due to their various higher value chemical applications in consumer packaged goods, surfactants, lubricants, and solvents.

The utilization of F-T to convert waste CO_2 and renewable H_2 to liquid fuels is hindered by the fact that the F-T reaction can only react CO with H_2 to produce diesel-type molecules with high yields. When CO_2 is fed in place of CO to a F-T-type reactor and catalysts, the result is high yields of undesirable and low value methane product, rather than more desirable and valuable liquid fuels such as diesel.[11,12] Research is ongoing into new compositions of metal-catalysts for F-T that will a) prevent the undesirable methanation reaction resulting in single-carbon methane product; and b) promote the desired production of multi-carbon hydrocarbons from H_2 and CO_2.[13] As an alternative to new and improved chemical catalysts, it has also been proposed that the standard reverse water gas shift reaction[14] be used to react renewable H_2 with CO_2 to produce CO, which can then in turn be fed, along with additional H_2, into the standard F-T reaction.[15] This is a feasible approach technically, but economic viability is uncertain. Such a pathway from CO_2 to liquid fuels may be viable in certain niche applications such as the onsite production of aviation fuels from CO_2 onboard nuclear powered aircraft carriers.[16]

The example of F-T is particularly illustrative in the context of the knallgas bioprocess platform that Kiverdi is attempting to develop. It represents the chemical counterpart and equivalent in terms of gaseous inputs (i.e. H_2/CO_2 gas mixtures or syngas) and product outputs (organic molecules with carbon numbers focused in the C8-C18 range). However rather than simply developing a biological version of F-T, Kiverdi is attempting to develop a process that is differentiated from F-T in a number of important ways including: 1) operating much closer to ambient conditions in terms of temperature and pressure; 2) having much higher tolerance of gas impurities such as sulfides and tars. It is predicted that these two characteristics can enable a large reduction in the capital costs compared to F-T, and widen the range of applicability and opportunities to apply the gas conversion technology. The process that Kiverdi is developing is also differentiated on the product side by targeting the slightly oxygenated oils and fats, such as fatty acids, which can be efficiently produced by biological organisms, but not by F-T. As

discussed these slightly oxygenated molecules generally command a higher price per unit weight or volume than the linear, deoxygenated hydrocarbons produced by F-T; due to their various higher value chemical applications. Furthermore, it is believed that a biological process can more efficiently target ringed, branched, or aromatic carbon-backbone molecules, through isoprenoid or aromatic amino acid biosynthesis pathways, then can chemical catalytic routes passing through either the F-T or MTG processes.

In addition to CO_2-to-methanol or CO_2-to-F-T hydrocarbons, there are many other different chemical approaches to the utilization of CO_2 that are being researched. Some have suggested using electrolysis to reduce CO_2 into simple low molecular weight products such as CO, methane, ethylene, methanol or formic acid[16]. Researchers have further shown interest in developing photocatalysts which use UV light to directly reduce CO_2 into CO[17]. None of these approaches directly produce liquid fuels or oils. As discussed above CO and methanol could feed into the F-T and MTG processes respectively, if multicarbon molecules such as liquid diesel or gasoline were sought after.

In addition to converting CO_2 to fuel-type molecules such as methanol or diesel, conventional chemical processing can also make other uses of CO_2. For example, RWE Power[18] describes a chemical process for using waste CO_2 as one of the precursor materials for the production of polyether polycarbonate polyols. This approach makes use of conventional chemical engineering processes, which are not yet directed to 'green' chemical processes.

1.3.3 The Ideal Hybrid Approach

Knallgas bioconversion technology draws on the best of both the abiotic and biological worlds - the ability of abiotic energy conversion systems to efficiently draw from any of a wide portfolio mix of energy sources – and not be limited to one particular energy source such as direct sunlight – combined with enzymatic capabilities gained through billions of years of evolution in fixing CO_2 - to produce the same types of oils that can be produced by plant oil crops or algae. This gives the hybrid chemical/biological approach much greater flexibility and geographic reach, as well as potential for scale-up, than fully biological CO_2-to-oil technologies based on photosynthesis in plants or algae.

In order to achieve the full potential of this technology, the following ideal characteristics in a knallgas strain and cultivation system are sought:

- Captures carbon from heterogeneous and contaminated gas streams.
- Obtains metabolic energy by oxidation of H_2 electron donor.
- Obtains metabolic energy by oxidation of CO electron donor (optional).
- Requires no other carbon input beyond CO_2 to grow.
- Significantly higher energy efficiencies than photosynthetic strains and systems.
- Carries extremely low risk of contamination.
- Does not require fresh water.

- Low water use and/or net water production (due to knallgas respiration).
- Does not require any complex nutrients.
- Hydrocarbons and/or organic chemicals synthesized in vivo.
- Naturally directs a high flux of carbon to the acetyl-CoA metabolic intermediate - which leads to fatty acid and terpenoid biosynthesis.
- Naturally produce a high quantity of triacylglycerol (TAG) through the fatty acid biosynthesis pathway (could be enhanced by metabolic engineering).
- Amenable to genetic modification.
- Non-pathogenic.
- Can't survive outside the bioreactor environment due to lack of H_2 nutrient - making organismal escape into the surrounding environment impossible.
- Hydrocarbons and/or organic chemicals secreted in culture (optional).
- Requires minimal downstream biomass processing.

CHAPTER 2:
Development of Microorganisms for Knallgas Bioconversion

2.1 Summary

This effort identified potential knallgas strains via strain screening procedures on $H_2/CO_2/O_2$ gas mixtures, characterize their product profiles, and evaluate growth and productivity at laboratory scale cultivation. Eight of eleven strains screened in serum bottles were grown successfully using $H_2/CO_2/O_2$ as the only substrates. Based on its robust growth properties, *Cupriavidus necator* was selected for follow-up studies in bench-scale bioreactors. The best run achieved with *C. necator* strain DSM 531 at this scale resulted in OD650 of 100 in four days and peak biomass production of 38 grams per liter dry cell density (130 OD) in six days.

2.2 Evaluation of Potential Knallgas Microorganisms

Knallgas strains were screened for growth on $H_2/CO_2/O_2$ and for production of lipids. Experiments were conducted in 160 ml and 240 ml serum bottles, 1-liter gas-tight HPLC bottles, and 500 ml to 2-liter bioreactors.

At the bottle scale, several knallgas species were tested for growth on $H_2/CO_2/O_2$ gas including *Cupriavidus and Rhodococcus* species. For facultative heterotrophic strains, an initial $H_2/CO_2/O_2$ grown serum bottle culture typically received a 5% by volume inoculation from a Luria broth (LB) grown culture. For obligate chemoautotrophs, a glycerol stock was directly inoculated into a serum bottle under $H_2/CO_2/O_2$ atmosphere. The initial serum bottle culture was grown between 72-168 hours following inoculation.

Experiments were generally performed on subcultures taken from the initial gas-grown serum bottle cultures above, typically using 5% inoculum by volume. Bottle growth on gas was performed in stoppered and sealed Wheaton glass serum bottles. A mineral salt medium (MSM[1]) was used that contained no organic carbon or complex nutrients. Certain strains required adjustments to the MSM, but none were given any complex nutrients or vitamins. Sterilized MSM was aseptically transferred into bottles, generally at a liquid to headspace ratio of 1:8, and the bottles were stoppered.

The $H_2/CO_2/O_2$ (or air as oxygen source) gas mixture was added at a targeted pressure to the bottles either by means of calculated syringe volumes of the individual gases or through flowmeters/mixers and a gas manifold. The hydrogen source was either from compressed gas

[1] Knallgas MSM: $Na_2HPO_4 \cdot 2H_2O$ 4.5 g/L, KH_2PO_4 1.5 g/L, NH_4Cl 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $NaHCO_3$ 0.5g/L, Ferric Ammonium Citrate (mfr, ID) 5 mg/L, $CaCl_2 \cdot 2H_2O$ 10 mg/L, $ZnSO_4 \cdot 7H_2O$ 100 µg/L, $MnCl_2 \cdot 4H_2O$ 30 µg/L, H_3BO_3 300 µg/L, $CoCl_2 \cdot 6H_2O$ 200 µg/L, $CuCl_2 \cdot 2H_2O$ 10 µg/L, $NiCl_2 \cdot 6H_2O$ 20 µg/L, $Na_2MoO_4 \cdot 2H_2O$ 30 µg/L. Thermophilic Bacteria, CRC Press, Boca Raton, FL, Jacob K. Kristjansson, ed., 1992, p. 87, Table 4.

tanks or generated in-house via electrolysis of water. Gas composition of the headspace was monitored with gas chromatography (GC) (Shimadzu GC-8A, TCD detector, helium carrier gas, and Alltech CTR I column). Following inoculation, the bottles were incubated with shaking, typically on their sides to enhance gas-liquid mass transfer.

It was found that most of the candidate knallgas strains tested could be grown chemoautotrophically on MSM and a gas mixture of 66.7% H_2, 23.8% air, and 9.5% CO_2 with CO_2 providing the sole carbon source for growth. Figure 7 shows an overview of the various tests and data collected during strain screening. Characterization and analyses included microscopy of cell morphology, description of colony morphology, cell mass composition, oil extraction and analysis, and growth behavior at the serum bottle through the bioreactor scale. Examples of cell morphology are shown in Figure 8. Wet mounts of the culture were observed using phase contrast optics with an Axioskop research microscope (Zeiss, Germany). Micrographs were generated with a MacroFIRE device (Optronics; Galeta, CA) using the PictureFrame (Optronics; Galeta, CA) software for imaging and data storage. Growth behavior was characterized by optical density (OD) measurements at 600 or 660 nm with a spectrophotometer (Beckman DU 640), cell-dry-weight, and ammonium utilization using a colorimetric method (API Ammonia Test Kit) to quantify ammonium in the culture broth. Other macro-nutrients, such as PO_4 and SO_4 were analyzed by ion chromatography (Dionex, ICS2000).

Figure 7: Overview of Analyses Performed for Strain Screening on $H_2/CO_2/O_2$ Gas Substrates

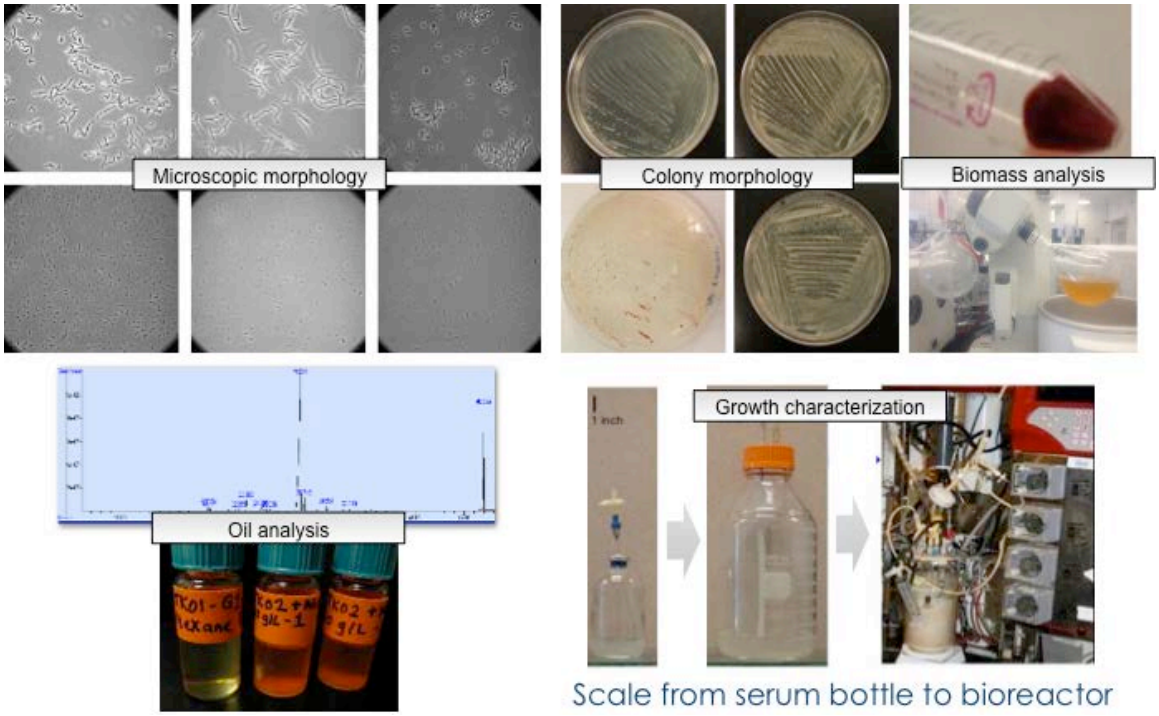
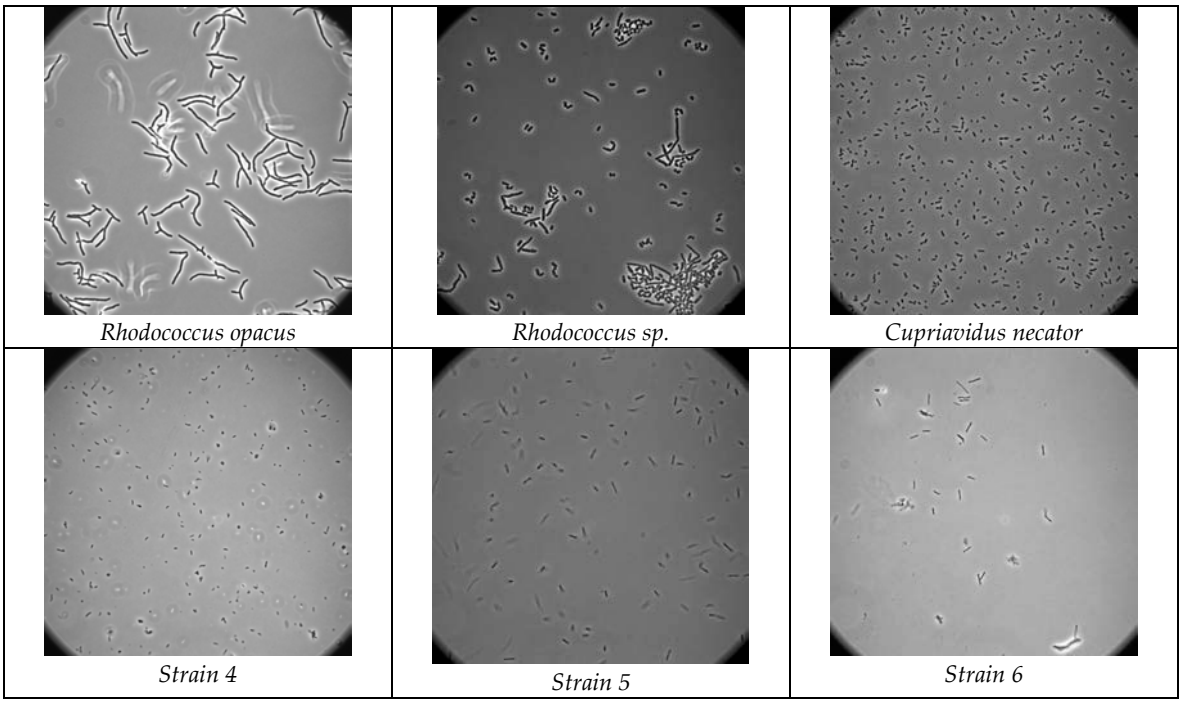


Figure 8: Microscopy of Knallgas Strains Grown on Gas



Biomass produced from $H_2/CO_2/O_2$ gas mixtures was used for gravimetric analyses that measured the total yield of cells, as well as the mass portion of lipids within the biomass. Gas chromatography/mass spectrometry (GC/MS) of derivatized lipid extracts was used to determine the composition of the fatty acids that were present.

Cupriavidus necator was among the most robust and fastest growing strains under the conditions tested, and was therefore chosen for more in-depth investigation at the bioreactor scale. The genus *Cupriavidus* produces high quantities, up to ~70% of cell mass[19], of the natural polyester poly-(R)-3-hydroxybutyrate (PHB). The ability of *C. necator* to accumulate high quantities of PHB is reflective of an ability to direct a high flux of carbon through the central metabolic intermediate acetyl-CoA, which, as discussed is also a metabolic intermediate of lipid biosynthesis. In initial proof-of-principal work, Kiverdi and other researchers in the field have demonstrated that *Cupriavidus* and related strains are amenable to metabolic engineering. Therefore it may be possible to redirect acetyl-CoA to fatty acid biosynthesis or other acetyl-CoA pathways (e.g. terpenes) instead of PHB synthesis. *Cupriavidus* and other closely related strains are the subject of several published scientific research and strain characterization studies, including the early NASA research[20]. *C. necator* is known to naturally fix CO_2 at a high rate under autotrophic conditions, as reflected by a fast growth rate on CO_2 as the exclusive carbon source (reported to be as low as two hour doubling times).[21] Other positive traits include a strong resistance to contamination through being able to out compete any contaminant strains that might enter the system by extremely fast and vigorous chemoautotrophic growth. This trait was suggested by early-published research on continuous runs with related strains that

remained productive for months without disruption[22]. This trait was confirmed in Kiverdi's laboratory as well and is discussed in later sections.

2.3 Laboratory Scale Bioreactor Development

2.3.1 Equipment, Set-up, and Protocol Development

A schematic of the bioreactor system with gas input lines is shown in Figure 9. Cultures were grown in custom-manufactured 500 mL glass or 2 L fermenters with PEEK headplates (Figure 10). Temperature and pH were controlled and monitored with a commercial controller (Electrolab, Fermac 360, United Kingdom). Temperature was controlled by a heating pad on the bottom of the reactor and a cooling water jacket on the reactor sides. The pH was maintained at 6.8 by adding only a base (either 2N NH_4OH or NaOH). The 500 mL fermenter sat on a stir-plate (VWR 12365-344) with a magnetic stir bar (cross shape, VWR 'spinplus' #58947-828). The stirplate was set to 200-400 RPM. The 2 L reactor had an agitator through the headplate, capable of rotating up to 500 RPM. A foam trap was provided on the vent line downstream of the condenser. Samples were withdrawn from a tube extending to the bottom of the reactor by means of an aseptic sampling system with 25 mL Schott bottles, using a procedure modified from instructions from colleagues at SRI International.

The gas supply was filtered house-air, compressed H_2 and compressed CO_2, each regulated to 20 psi. H_2 and air were delivered to a flow proportioner (Matheson G2-4D151-E401/E401, 20 psi), which sets the relative fraction of the gases. CO_2 was delivered to a variable area flow meter.

Figure 9: Configuration of Bioreactor Process with Gas Lines

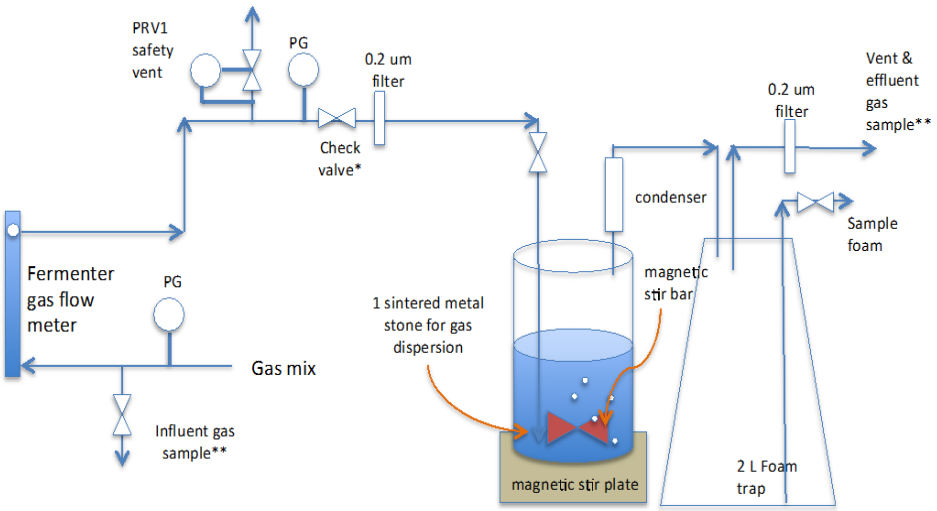
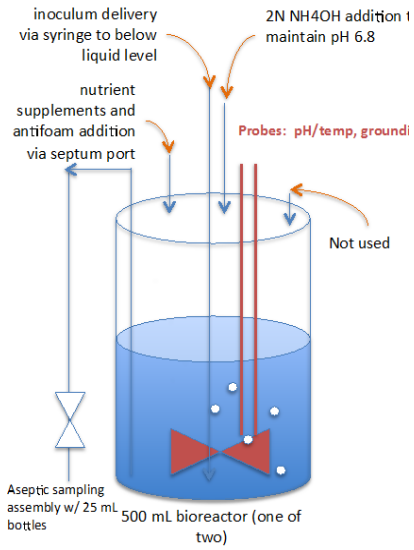


Figure 10: Laboratory Scale Bioreactor Detail



The H_2/air gas mix from the flow proportioner was connected into the CO_2 gas line, and then delivered to each bioreactor through a variable area flow meter. A pressure gage was used to monitor the gas delivery pressure to the bioreactor. Gas was mixed into the bioreactor broth; different dispersion stones were used during the course of the tests, including 2-micron diffusion stones (p/n KEG592, http://morebeer.com/products/diffusion-stone-2-micron-oxygen.html), or two porous pyrex frits (40-60 um, Sigma-Aldrich CLS3953312-C) when a high-salinity medium was tested. Size 0.2 micron filters (Pall, p/n 4251) were installed upstream of gas entry to the reactor and downstream of the foam-trap. Gas was vented to an exhaust system.

Oxygen concentration in the gas delivery and vent lines was monitored periodically with a fiber-optics probe (Fospor-R, Ocean Optics, with NeoFox spectrophotometer). Samples from the gas delivery and vent lines, collected in gas-sampling bags (Flexifoil sample bag 262-01, SKC Inc.) were analyzed by GC to confirm the settings of the gas flow valves.

The composition of the effluent and influent gas streams was also used to determine uptake rates, mass balances, and mass transfer efficiencies. The concentrations of NH_4, PO_4 & SO_4 were monitored frequently to determine:

- Deficiencies that could have hindered bacterial growth.
- Conditions that triggered lipid accumulation.
- Which time points during the bioreactor runs certain of these key compounds needed to be replenished or allowed to be completely consumed in order to optimize the biomass and lipid yields.

Lab-scale cell mass dewatering, drying, and oil extraction techniques were tested to determine the most appropriate and scalable methods.

Base bioprocess protocols were finalized through preliminary experiments. Key elements that were tested and finalized included the following:

- As gas was pumped through the reactors, small bubble size was required for enhanced mass transfer. The final reactor design used four diffusion stones per reactor, which was observed to improve gas transfer.
- Proper plumbing was key to obtaining high cell mass. The system was re-plumbed several times to ensure proper gas flow and control. Modifications were made to the bioreactor setup to ensure proper gas delivery so that two to four bioreactors could be run in parallel using the same gas supply. Multiple reactors allowed for duplicate testing of the same operating conditions or experiments with targeted single parameter variation.
- A protocol was developed to measure gas-phase H_2, O_2 and CO_2 concentrations using GC, to verify gas flow settings and oxygen probe calibration.
- Foam-control measures were used to reduce required antifoam volumes that included timing and type of antifoam addition, mechanical means of foam control and overcoming certain nutrient deficiencies that caused foaming.
- Nutrient addition strategies were tested to finalize a base nutrient level optimal for growth as a function of process parameters, including rate of OD change and lipid accumulation.
- The increase of gas/liquid mass transfer and biomass growth rate at elevated pressures was established.
- Inoculum growth was optimized, described in more detail below.

2.3.2 Bioreactor Growth of *C. necator* on $H_2/CO_2/O_2$ Gas Mixtures

Inoculum for bioreactor runs was prepared by growing *C. necator* DSM 531 on $H_2/CO_2/O_2$ in serum bottle culture to OD ~1. The bioreactor was inoculated to OD ~0.1, and pH was controlled with 2N NH_4OH. A protocol was developed for the addition of mineral nutrients (other than NH_4^+ which was provided through pH adjustment) during culture growth in response to certain measured cues, so as to ensure that none of the major and trace mineral nutrients were growth-limiting. Initial runs using this mineral nutrient supplementation protocol resulted in chemoautotrophic growth of C. *necator* on exclusively gaseous carbon and energy substrates ($H_2/CO_2/O_2$) to an OD650 of 89 in six days (Figure 11, condition A). Subsequent modifications to the protocol included addition of antifoam to control foaming and continuous liquid recirculation and spraying at 280 mL/min to enhance gas/liquid mass transfer. The best run achieved with *C. necator* strain DSM 531 at this scale resulted in OD650 of 100 in four days and peak biomass production of 38 grams per liter dry cell density (130 OD) in six days (Figure 11, condition B). The recovered biomass was analyzed for lipid content and distribution. Under

typical conditions used, *C. necator* produced total fatty acid content of ~10% of the dry cell weight. The major lipid species present were palmitic acid (C16:0, 30-60%) and palmitoleic acid (C16:1, 10-15%) (Figure 12), with the highest levels of C16:0 occurring in earlier time points in the run. Significant levels hydroxylated oleic acid (C18:1) species were also identified.

Figure 11: Growth Profile of *C. necator* under Two Different Operating Conditions

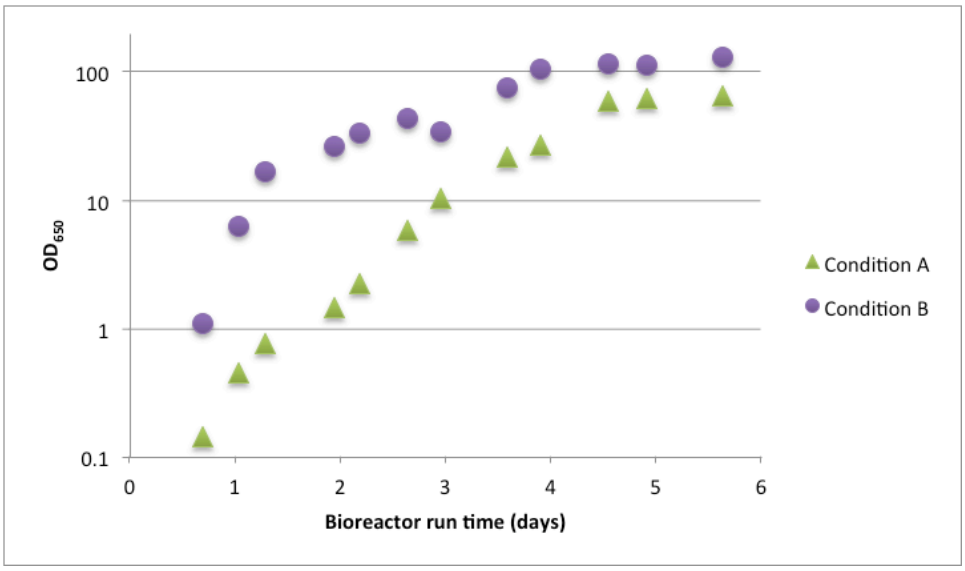
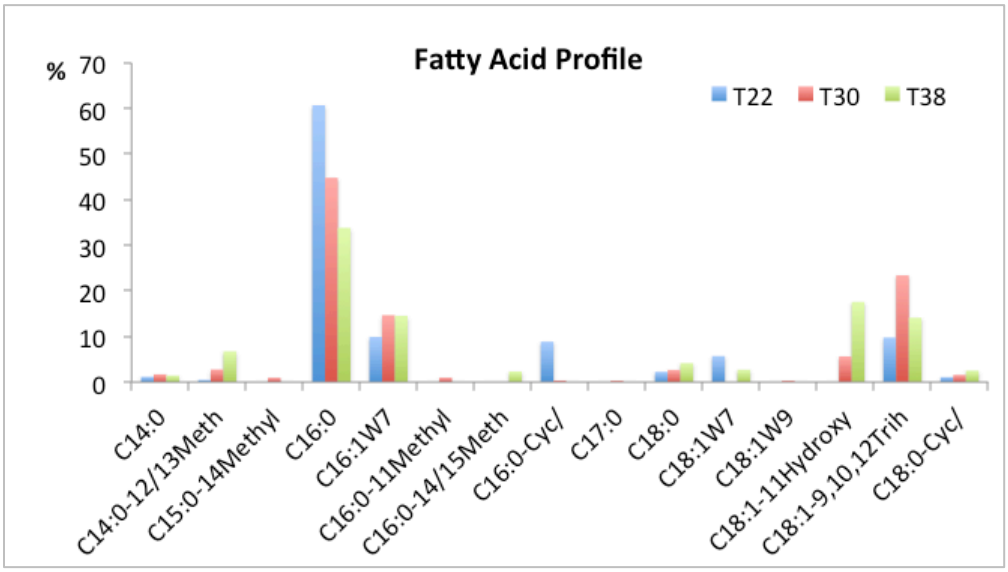


Figure 12: Fatty Acid Profile of *C. necator* from Bioreactor Growth



2.4 Analysis of H_2 Utilization by *C. necator*

2.4.1 H_2 Utilization from Different Industrial Sources

Kiverdi identified and evaluated sources, technologies, and methods for the generation of H_2 that can be utilized in the CO_2 bioconversion process. The different sources of H_2 identified were evaluated in terms of renewability, sustainability, greenhouse gas impacts, feasibility, compatibility with the process, scalability, cost, and overall near term and long term potential. Lab testing was performed on three types of H_2 containing gas generated or captured from renewable or waste sources:

1. A H_2 and CO containing tail gas taken from a chemical process converting methane to ethylene.
2. H_2 and CO containing gas produced by a small gasifier from a wood and agricultural residue (walnut shells) feedstock.
3. H_2 generated by electrolysis from tap water using electric power provided by PG&E.[23]

Strains were confirmed to have tolerance to various contaminants present in the various hydrogen containing gases that were tested and analyzed. The ability of the strain to tolerate and grow on hydrogen containing gases produced (1) as a tail gas from a chemical process; (2) by a gasifier; and (3) by electrolysis; demonstrates the flexibility of the bioprocess to utilize a wide variety of energy and hydrogen inputs for the capture and conversion of CO_2 to oils and other valuable biochemicals. This flexibility in the source of H_2 input is key to tapping into abundant, sustainable or waste sources of energy and carbon, as feedstocks for the biochemical conversion of CO_2 to oleochemicals, ranging from solar, wind, or geothermal power, to waste biomass or industrial tail gases.

2.4.2 H_2 Utilization Efficiency

To assess the amount of biomass produced per H_2 consumed by a knallgas strain, a preliminary mass balance experiment was performed based on the approach of Lynd and Zeikus[24]. The experiment was conducted in an effort to test whether higher biomass production per gram of H_2 consumed is observed in a knallgas strain versus hydrogenotrophic acetogenic microbes, as predicted by the Rittmann McCarty (R&M) bioenergetics model[25]. In the following experiment, *C. necator* strain DSM 531 produced approximately four times more cell mass per gram of H_2 consumed than has been reported for a hydrogenotrophic acetogen. Higher cell mass synthesis per H_2 consumed is consistent with superior efficiency in ATP production and anabolic biosynthesis.

Gas-tight 160 mL serum bottles (as described in Section 2) containing 19 ml MSM were inoculated with *C. necator* strain DSM 531. After sealing the bottles, a gas mixture (66.7% H_2, 23.8% air, and 9.5% CO_2) was added at 15 psig to the bottles through a manifold. The bottles were then oriented horizontally in a shake flask incubator (MaxQ™ 5000 Floor-Model Shaker, Thermo Scientific) set at 30°C and 250 RPM. The sixteen serum bottles included 14 experimental replicates and 2 negative controls. The negative control bottles had identical preparation as

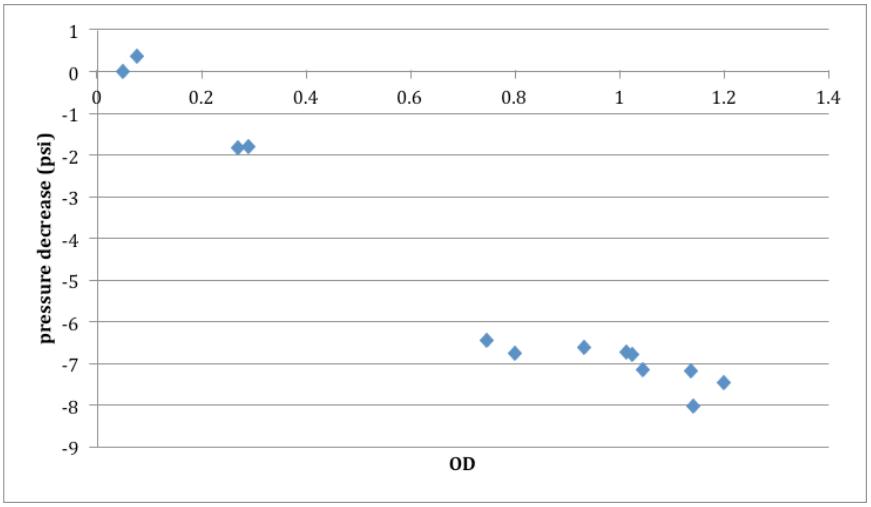
experimental bottles minus the inoculum, and were used to detect any contamination and/or abiotic loss or leakage of gas from the bottle headspace.

At each time point, one to three experimental replicate bottles were sacrificed for analysis. An experimental and a negative control bottle were sacrificed at T0 for data at the beginning of the experiment. The second negative control was sacrificed at the final timepoint. Gaseous consumption within the serum bottles was measured using a pressure gauge connected to a needle. The headspace gas pressure was measured for each sacrificed bottle, and a 100-uL sample of headspace gas was taken by gas-tight syringe for gas chromatography (GC) analysis. Gas headspace content of H_2, CO_2, O_2, and N_2 in the serum bottles was quantified at each time point using GC (Shimadzu GC-8A, TCD detector, helium carrier gas, and Alltech CTR I column). H_2 concentration was determined by mass balance from the measured O_2, CO_2 and N_2 concentrations. An estimate of CO_2 dissolution into solution was made from the measured CO_2 headspace content in the T0 control, and the CO_2 headspace content in the negative control sacrificed at the end of the run.

For sampling the broth, the septum of serum bottle was wiped with a 70% ethanol solution and the entire liquid contents of bottle withdrawn into a 30 mL syringe using bottle pressure. A 100 µL aliquot of sample was pipetted out for OD measurement at 650 nm. When sufficient biomass was produced, as indicated by OD, cell dry weights were determined. Samples collected for dry weight determination were first centrifuged at 12,000 G for 15 min at 4°C. Pellets were resuspended in 10 mL sterile PBS, vortexed, and vacuum filtered through pre-weighed 0.45 µm filters. Prior to filtration, the membrane filters were pre-dried at 60°C then cooled in a desiccator to room temperature, after which the pre-weighing was performed. Following filtration, the filters were dried at 60°C for 24 hours, cooled to room temperature, and the filter + biomass retentate was weighed to determine biomass dry weight. This cycle of drying and re-weighing was continued until the weight remained constant. This constant weight determined for the sample was recorded as the cell dry weight. A correlation was developed between OD and biomass density (dry cell weight per volume).

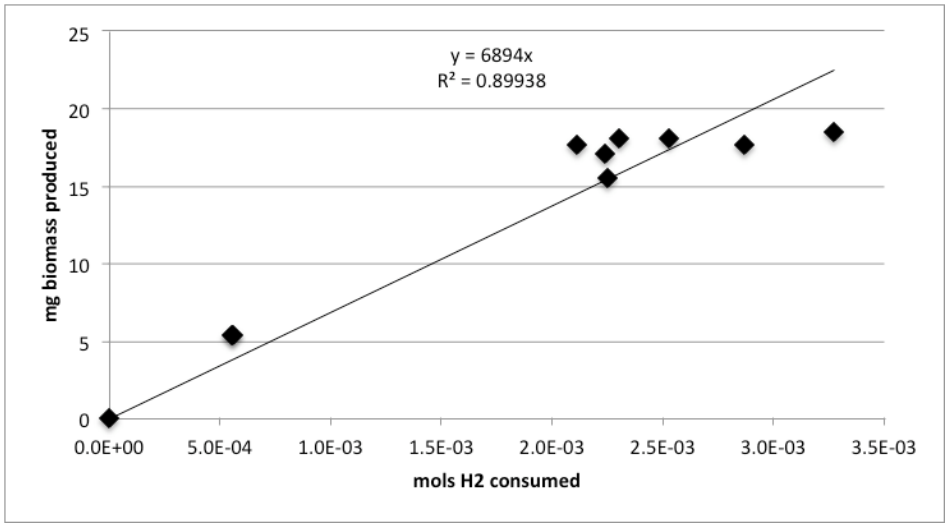
The decrease in headspace pressure was associated with the increase in OD as shown in Figure 13. This decrease is due to the consumption of gases (i.e. H_2, CO_2, and O_2) by the knallgas microorganisms for the production of cell mass.

Figure 13: Decrease of Headspace Pressure as a Result of Growth in Airtight Bottles



Assuming the ideal gas law (PV = nRT) for the headspace gases, the total moles of gases were calculated, accounting for temperature variation in sample points. Using the measured gas headspace composition and pressure, and the measured dry weights, the proportion of cell weight to moles of H_2 consumed was determined (Figure 14).

Figure 14: Biomass Yield per Moles of H_2 Consumed



These results indicated that between 6.7 to 7.1 grams of dry cell mass were synthesized per mole of H_2 consumed, or 3.3-3.6 grams cell mass per gram of H_2. In a similar experiment performed on a hydrogenotrophic acetogen, *Butyribacterium methylotrophicum*, Lynd and Zeikus found the yield of dry cell mass to be 1.7 g /mol H_2 (0.85 g cell mass / g H_2).[26] The result — roughly four times higher cell mass per H_2 for the knallgas organism *C. necator* compared to an acetogen — is consistent with what is predicted by the R&M bioenergetics model[27]. Note, according to the

R&M model, this difference in cell mass yield results from the thermodynamics of respiration, and is not strain dependent, beyond the ability or inability a given strain to utilize a given redox couple in respiration. Therefore, this result is expected to hold generally across many different knallgas strains and many different acetogenic strains. For acetogens, and anaerobes generally that use CO_2 as an electron acceptor, the majority of the carbon and electrons flow into C1-C4 products such as acetic acid, ethanol, or methane, which diffuse out of the cells and dissolve into solution or the gas headspace. Consequently these small molecules do not contribute to the measured cell mass. Conversely, for knallgas microorganisms, the majority of the carbon and electrons flow into the products of anabolic biosynthesis such as lipids, proteins, polysaccharides, and other biopolymers. The products of anabolic biosynthesis accumulate intracellularly and comprise almost the entirety of the cell mass. Consequently the measured cell mass represents the total output of anabolic biosynthesis.

From the experiment determining biomass produced per H_2 consumed, an equivalent ratio for the consumption of CO_2 was determined as well. To accurately determine consumption of CO_2, the relatively high solubility of CO_2 into the media had to be accounted for. This was done by experimentally determining a Henry's constant for CO_2 in the media used at the temperature the experiment was performed. Due to the dissolved minerals in the solution, this was found to differ significantly from the Henry's constant for pure water. It was determined that 0.61-0.65 grams of biomass were synthesized per gram CO_2 consumed.

2.5 Conclusions

The following key results were produced by the experiments performed:

- Multiple knallgas strain candidates were identified, and shown to grow on a CO_2 and H_2 substrate as sole carbon and energy source.
- Robust protocols for propagation and cultivation of knallgas microorganisms were developed. In particular, Kiverdi successfully defined a set of Standard Operating Procedures (SOPs) for reproducible generation of a high cell mass using CO_2 as the sole carbon source. This was accomplished by performing multiple bioreactor runs in parallel (as described above). Existing protocols were fine-tuned and proven to be robust for achieving a base bioprocess upon which to build in order to achieve commercialization metrics.
- The final SOPs enable higher throughput for the gas bioprocess experiments and the demonstrated reproducibility of achieving high cell mass with the microbes when grown on CO_2.
- Successful upscaling of the growth of *C. necator* to 2 L bioreactor scale and achieving 38 g/L peak biomass concentration in 6 days.
- Efficient H_2 utilization by *C. necator*, ~4X greater cell mass production per H_2 consumed than hydrogenotrophic acetogens, was demonstrated. This reflects superior efficiency in ATP production and anabolic biosynthesis.

CHAPTER 3:
Continuous Cultivation on Gaseous Substrates

3.1 Summary

The objective of this work was to convert the batch lab-scale gas bioprocess to a continuous culture bioprocess on $H_2/CO_2/O_2$ gas mixes with *C. necator*. In particular, the medium composition, the operational conditions (pH, temperature, gas flow rate, gas composition), and protocols for system inoculation, sampling, harvesting and analysis of product, were based upon the SOPs discovered and developed in the batch runs performed in the smaller bioreactors described in the previous chapter. The cell density, lipid content, and biomass/lipid productivities were determined for continuous-flow runs. This work also produced larger samples of biomass for oil extraction. The work performed in this chapter, together with large-scale batch experiments in the following chapter, helped determine operational parameters necessary to initiate engineering and design of an integrated and continuous skid-mounted pilot plant. Continuous culture bioreactor experiments described here were performed at Lawrence Berkeley National Laboratory (LBNL).

3.2 Equipment and Experimental Set-up

A continuous flow reactor system to grow knallgas microbes and remove produced cell mass from the bioreactor was designed in collaboration with LBNL partner, Dr. Jil Geller. This design work entailed converting the protocols developed for batch bioreactor runs to protocols for continuous production runs. The conversion from batch runs to continuous production runs was part of the overall project objective of enhancing and improving the process for the biological conversion of CO_2 into oils. The resulting design for a continuous flow reactor system enabled a higher throughput of inputs and higher rates of product generation. A detailed protocol for operation of the continuous system was developed, as well as adapted various analytical methods and laboratory protocols to this continuous system.

Due to the relatively low conversion rate by the microbes of gases, such as H_2, and its comparatively poor aqueous solubility, each time the gases pass through the working volume of the reactor, a relatively small proportion of the gas is actually consumed. Consequently, a gas recirculation system was developed for this continuous process in order to reduce the total gas requirement. A schematic of the gas recirculation system and the liquid flow system used in the continuous process can be seen in Figure 15 and Figure 16, respectively. Using this set-up, the team demonstrated continuous processing of H_2/CO_2 gas mixtures into oil products based on the bioreactor-scale process described in the previous chapter, using the knallgas strain *C. necator*. Continuous runs on the order of 100 hours in duration were performed, with continuous harvesting of cell mass. Continuous harvesting of the cell mass was implemented through the continuous removal of cell broth, the transfer of harvested broth to cooled storage, and the continuous replenishing of medium, to replace the liquids and nutrients lost in cell broth removal. Microbial oils were, in turn, extracted from the harvested cell mass through sequential, batch, solvent extractions from the harvested cell mass.

Figure 15: Continuous Process Gas Recirculation System

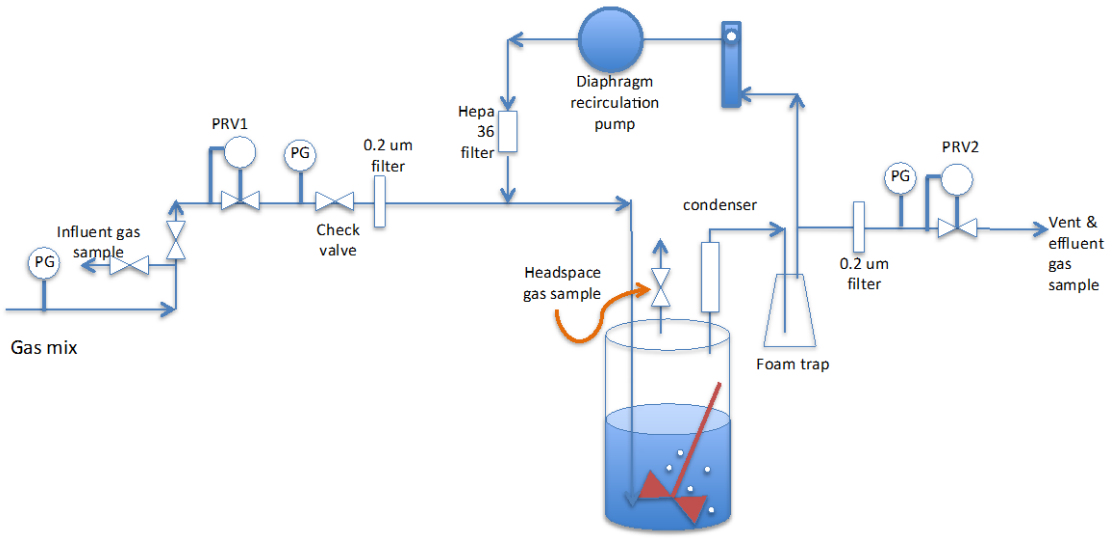
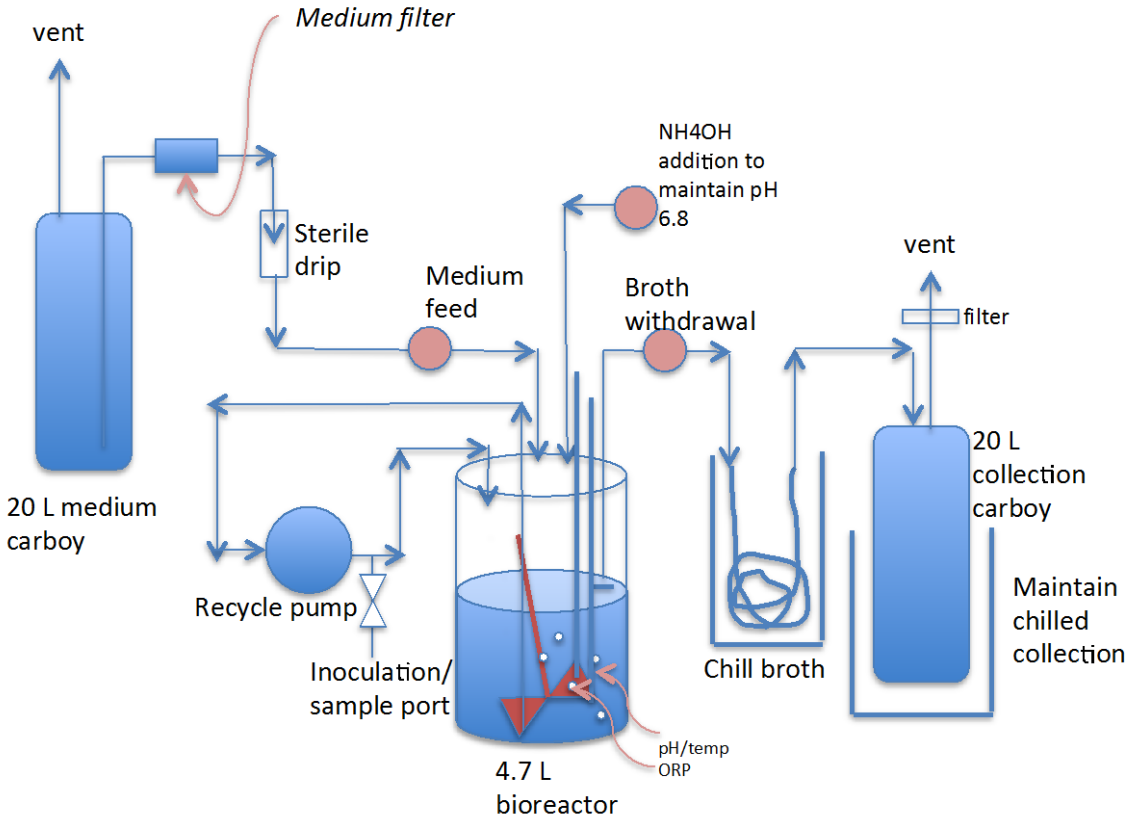


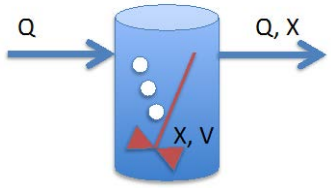
Figure 16: Continuous Process Liquid Flow Lines



3.2.1 Growth Kinetics in Turbidostat

The type of continuous flow bioreactor system used for this project is referred to as a turbidostat. A turbidostat is a continuous-flow stirred-reactor, or CSTR, having feedback between the turbidity (or OD) of the culture and the dilution rate (or flow rate). A schematic illustrating the major operating principles of a turbidostat is shown in Figure 17.

Figure 17: Schematic of Turbidostat Continuous Culture Operation



Q is the volumetric liquid flowrate (where the influent flow rate entering the reactor must equal the outward, effluent flow rate leaving the reactor), V is the liquid volume in the reactor, and X is the bulk biomass concentration inside the reactor. Because the reactor is stirred, the effluent biomass concentration is also equal to bulk concentration in the reactor (X). Assuming exponential growth, mass balance (neglecting death rate of cells) of the biomass in the system can be calculated as:

$$V\frac{dX}{dt} = V\mu_{log}X - QX$$

where μ_{log} is the growth rate during the exponential growth–phase. At steady-state, the concentration does not change, so dX/dt=0. Eliminating the time dependent variable, the equation simplifies to:

$$Q/V = \mu_{log}$$

The ratio Q/V is referred to as the dilution rate (D), which the above equation shows is equal to the exponential biomass growth rate. The biomass doubling time during exponential growth is equal to $\ln(2)/\mu_{log}$.

In addition to exponential growth, knallgas cultures can exhibit a prolonged linear growth phase, observed in some cases under batch growth conditions. For linear growth, the equivalent mass balance equation is:

$$VdX/dt = V\mu_{lin} - QX$$

and thus, just as above, the time dependent variable drops out at steady-state resulting in:

$$Q/V = \mu_{lin}/X$$

Note that the rate at which biomass is harvested from the bioreactor, whether exponential or linear-type growth, equals QX.

3.2.2 Details of Set-up and Operation

The bioreactor system, equipment, and protocols used for batch runs described in the previous chapter were modified to perform continuous runs, as shown schematically. One modification was the use of a larger 5 L glass bioreactor (Electrolab, Fermac 360, United Kingdom) with a working volume of 4.7-liter, and a PEEK (Polyether ether ketone) headplate and agitator that could be rotated at rates up to 500 RPM. A liquid recirculation pump provided mechanical foam control, as well as enhanced gas/liquid mass transfer, by either spraying broth from the top of the headspace onto the foam, or in reverse flow by removing foam from the headspace and returning it to the broth at the base of the liquid working volume. Fresh input medium for running the continuous flow was sterilized prior to introduction into the bioreactor by pumping through a 0.2 μm filter (Polycap 75 AS, cat. No. 6705-7502, Whatman). The fresh medium input tube in the reactor was submerged below liquid level to ensure adequate mixing and to minimize short-circuiting to the broth-outlet tube, which was positioned at the liquid level. A sterile drip was included in the input media line to prevent bacteria from migrating from the reactor back into the filter. Temperature and pH control were performed as described for the batch reactor (Section 2.3.1). An oxidation-reduction potential probe (ORP) was used for continuous monitoring of the redox state of the broth.

For the larger 4.7-liter working volume, it was estimated that 0.7 hydrogen gas tanks (261 cu. ft. per tank at STP) per day was required to maintain a flow rate of 1 VVM H_2. To reduce this rate of gas consumption, gas recirculation was implemented as diagrammed in Figure 3.1 and a gas flow rate through the reactor of less than 1 VVM was generally used. A diaphragm pump was used to recirculate reactor headspace gases. It delivered a maximum flowrate of 2.9 LPM at a reactor-headspace pressure of 1 psi, equivalent to 0.66 VVM, assuming a reactor working volume of 4.4 L. The mixture of compressed H_2, compressed CO_2, and house air, as described in Section 2.3.1, was sparged into the bioreactor broth via a glass frit below the agitator, and vented from the reactor through a condenser to a 2 L foam trap, consisting of a 2 L medium bottle fitted with an HPLC valve cap. All elements between the 0.2 μm filters (Pall, p/n 4251), and including the filters, were autoclaved with the reactor for 40 minutes prior to use in a continuous run. The gas recirculation lines, pump and flowmeter that were not autoclavable were flushed with 70% ethanol and dried by pumping air filtered to remove bacteria (0.2 μm filters). Gas pressure was controlled by PRV1 on the influent line, set to 10 psig, and PRV2 on the vent line, set to 1 psig.

Providing the 10% inoculum to the larger bioreactor used in the continuous runs, required around 470 mL of inoculum for the 4.7-liter working volume of one reactor. This was difficult to provide through growth of inoculum in serum bottles. To overcome the difficultly in producing an inoculum of sufficient size for the ca. 4.7 liter bioreactor, growing the inoculum on sugar instead of gas was tested. It was found that the *C. necator* strain can readily transition from growth on sugar to growth on gas with minimal lag time, and henceforth the use of sugar grown inoculum was utilized due to greater convenience in rapidly producing sufficient inoculum for the continuous flow bioreactor. Previously with batch runs, sugar-grown inocula were washed in order to prevent unwanted sugar nutrients from being carried over into the experiment. However in the case of continuous flow, sugar is removed by the continuous

delivery of basal medium, which dilutes and washes away any carry over sugar nutrient from the inoculum.

It was estimated that the continuous system would produce 0.83 L/hr of culture broth (the aqueous mixture of cells and liquid media flowing out of the bioreactor) and 0.83 L/hr x 24 = 20 L would be produced every 24 hours. Produced broth was collected in a chilled 20 L carboy, and centrifuged in one batch per day to recover the cell mass.

Fiber-optic dissolved oxygen (DO) probes (Foxy probes, Ocean Optics) was used which work at low DO levels and do not suffer from interference with H_2 as is the case with standard Clark-type DO probes. To reduce signal degradation from autoclaving, the probe used in the bioreactor (Hioxy T1000 probe, Ocean Optics), was sanitized in a 70% ethanol solution and inserted after the reactor was autoclaved. A two-point calibration of the DO probe was performed in the reactor before inoculation by sparging the reactor medium with nitrogen gas at the operating temperature of 30°C, for the zero oxygen calibration point, and then with air (20.9% O_2), for the second calibration point. Additionally a fiber-optic DO probe (Foxy Fospor, Ocean Optics) external to the bioreactor was used to measure DO on samples withdrawn from the reactor. However a difficulty with this approach was that the DO levels and the temperatures of the samples began to change immediately upon removal of the samples from the bioreactor. The DO values and temperatures in the samples were found to drop over the first approximately 10 minutes. This drop was caused by removal from the gas sparging system and the ongoing oxygen consumption by the *C. necator* microorganisms in the sample, combined with removal from the temperature control of the bioreactor. Consequently the DO value in the reactor measured by DO probe had some uncertainty and only provided relative information (i.e., relatively higher or lower DO from a base case).

Medium flow-rates to the reactor were adjusted to maintain constant OD, measured in broth samples obtained with the aseptic sampling assembly described in Section 2.3.1. Since continuous flow runs of long duration have increased risk of contamination, the team included a schedule for sampling and checking for contamination during the continuous run.

Testing of different operating conditions was performed and the effect on OD and growth rate, as indicated by the dilution rate D = Q/V, was measured. As previously discussed, D equals μ_{log} under exponential growth conditions, and μ_{lin}/X under linear growth conditions, where X is determined from the linear correlation between OD and dry biomass density.

3.3 Continuous Culture of *C. necator*

3.3.1 Continuous Culture Run #1

The first continuous flow test in a 4.7 L bioreactor (Figure 18) was inoculated with a *C. necator* culture in the late-stationary phase grown on LB to give an initial reactor OD of 0.05. An additional 800 mL of medium was pumped into the initial volume of 3.9 L, and batch growth was monitored until the operational OD was attained.

The input gas composition throughout the run was: $4.8\% < pO_2 < 6.4\%$; $7.2\% < pCO_2 < 9.9\%$, $62\% < pH_2 < 68\%$. The make-up gas mixture was provided at a rate of 0.3 to 0.5 VVM and the

volume of gas run through the reactor by the recirculation system was 0.6 to 0.7 VVM. The replacement liquid medium flow began on Day 5, at a rate ranging from 1.7 to 2.4 L/day. The reactor working volume was maintained between 4.4 and 4.7-liters. A steady-state OD was maintained at around OD 10. It was found that the continuous broth withdrawal helped with foam control compared to the batch system by removing foam from the liquid surface as it formed. The dilution rates, and hence growth rates, were indicative of linear growth kinetics as opposed to exponential growth (see Section 3.2.1). Some important metrics from the first continuous run are given in Table 3.

Figure 18: *C. necator* Growing on H_2 and CO_2 in Continuous Culture (Turbidostat) Operation

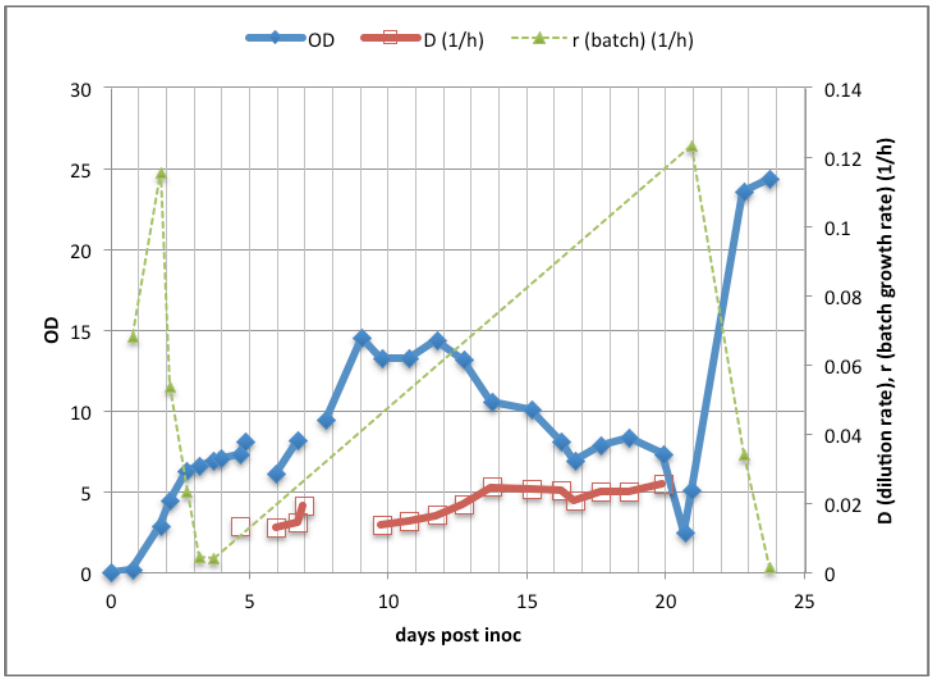
Table 3: Results from Continuous Culture Run #1

Total volume of broth harvested (L)	AVG OD	Run duration (days)	Total wet weight of harvested biomass (g)
45.2	11.12	19	786

During the continuous run, the maximum exponential growth rate observed (calculated from two sequential OD readings) was 0.12 hr^{-1} (Figure 19). This growth rate occurred during the initial and final batch growth periods prior to, and at the end of, the continuous flow of media through the system. Averaging over the first four points of the growth curve, the initial batch period growth rate was 0.09 hr^{-1}, slightly lower than that calculated using the 2nd and 3rd points alone. The exponential growth rates observed in eight batch runs of *C. necator* in 400 mL reactors ranged from 0.063-0.18 hr^{-1}, with an average of 0.12 hr^{-1}, and standard deviation of 0.04 hr^{-1}. Consequently the exponential growth rate observed using reactors at 10X larger scale was consistent with what was observed at the smaller scale. However, during the continuous flow

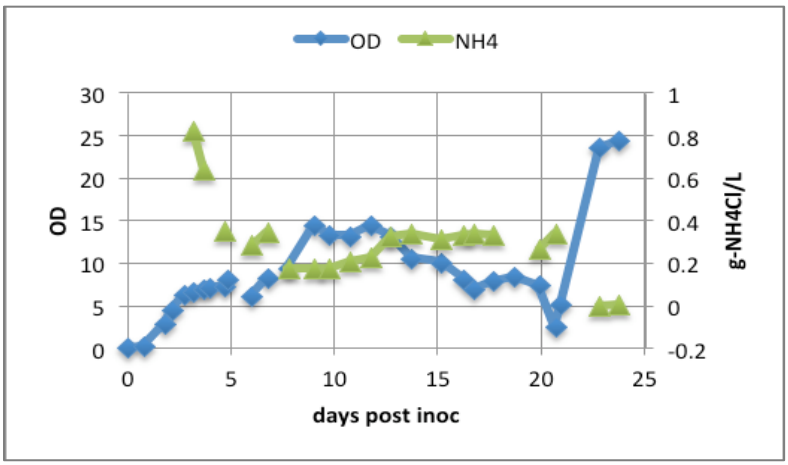
phase, D, the dilution rate, was 0.025 hr^{-1}, far lower than the expected dilution rate for exponential growth. According to the linear growth model, assuming an average OD of 10, it would be expected that μ_{lin} = D*OD = 0.025*10 = 0.25 OD/hr. The linear growth rates observed previously in batch runs of *C. necator* in the smaller 400 mL reactors ranged from 0.08 to 1.3 OD/hr, with an average of 0.4 OD/hr and an SD of 0.45 OD/hr. Hence the D that enabled a steady state of OD~10 was more consistent with the culture being in a linear type growth phase than an exponential growth phase. On the basis of this slower than expected growth rate, the estimated oil production in the first run, assuming 5% neutral lipids by weight, was 0.84 g-oil/day, compared with the predicted average of 4.1 and 1.3 g-oil/day for exponential and linear growth models, respectively.

Figure 19: Continuous Culture Run #1 ODs, Batch Phase Growth Rates(r) and Continuous Phase Dilution Rates (D)



Ammonium (NH_4^+) concentrations were measured during the course of the run and were found to be fairly steady through the continuous flow phase (Figure 20). The NH_4^+ concentrations were negatively correlated with OD in the batch phases of the run, as the culture consumed NH_4^+ with growth. At the end of the final batch period, after the flow of fresh media was turned off and where ODs approached 25, the culture was depleted with respect to NH_4^+.

Figure 20: Continuous Culture Run #1Ammonium (NH_4+) Concentrations



The records of pH and the culture redox potential (ORP) during continuous culture run #1 are shown Figures 21 and Figure 22. Dissolved oxygen was not continuously measured in this run. The fluctuation in ORP could be in response to changing dissolved oxygen (DO) levels, both due to gas flow, as well as fresh medium delivery (which was aerobic) supplying oxygen to the broth. However the relationship between ORP and DO is complex, as ORP may be affected by other redox-active components (e.g., H_2, iron, etc.) and pH, as indicated by the trends.

Figure 21: Continuous Culture Run #1 Redox Potential and pH

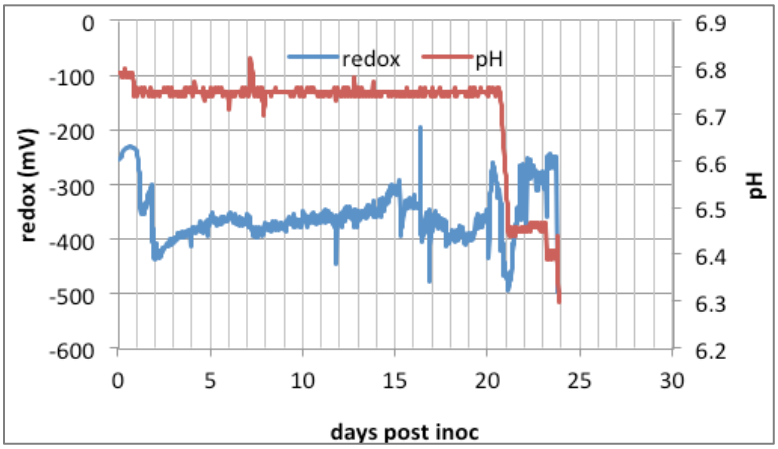
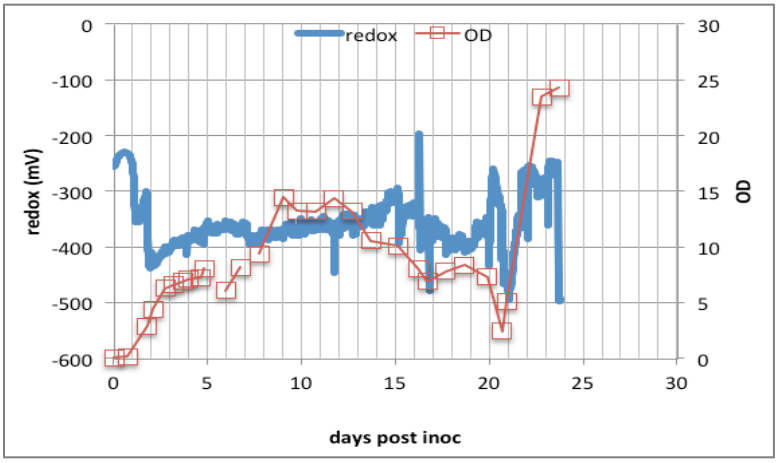


Figure 22: Continuous Culture Run #1 Redox Potential and OD



3.3.2 Continuous Culture Run #2

The following modifications were implemented for the subsequent continuous flow run:

- Dilution rates and gas-supply rates were adjusted to provide conditions favorable to exponential growth kinetics.
- Continuous nutrient amendment to replacement medium was provided during continuous flow, instead of adding incremental, proportional doses.
- The fiber-optic DO probe was installed in the reactor for continuous DO monitoring.

The culture was inoculated to OD 0.05 and grown in batch mode until it reached OD ~6. Medium was then added to fill the reactor to a working volume of 4.4 L, and the culture grown in batch to OD 6. The medium flow-rate was then set to match the growth rate measured at OD 6 in the batch growth phase. An OD of 6.6 was maintained at a dilution rate of 0.03 hr^{-1}, and 1.1 VVM total gas supply (including 0.6 VVM from gas recirculation and make-up gas at a rate of 0.5 VVM). This dilution rate is in the range of linear growth-rate constants, based on the analysis of previous *C. necator* batch reactor tests.

Following a period of maintaining the bioreactor at a steady state around OD 6.6, a new steady state of OD 1.8 was initiated by increasing the dilution rate to 0.09 hr^{-1}. The gas flow was set at 0.5 VVM without gas recirculation. This higher dilution rate falls in the range of exponential growth rates determined in the analysis of small-scale batch-runs. An increase in gas flow to 0.6 VVM resulted in an increase of OD to 2.0 for the same dilution rate of 0.09 hr^{-1}, suggesting that growth was limited by a nutrient in the gas supply. ODs, growth rates from batch periods (r), and dilution rates from continuous run periods (D=medium-flow-rate/reactor-volume) throughout the second run are shown in Figure 23. Observation of contaminants in plates of broth samples are noted in the figure, although contaminants were small in number. The growth rate (r) is calculated for exponential growth using OD of the current and preceding time-point. A roughly linear negative correlation for the values of r and D with OD was

observed as seen in Figure 24. Values of D fall within the same trends as r, indicating D is a good representation of growth rate.

Figure 23: Continuous Culture Run #2 ODs, Batch Phase Growth Rates(r) and Continuous Phase Dilution Rates (D)

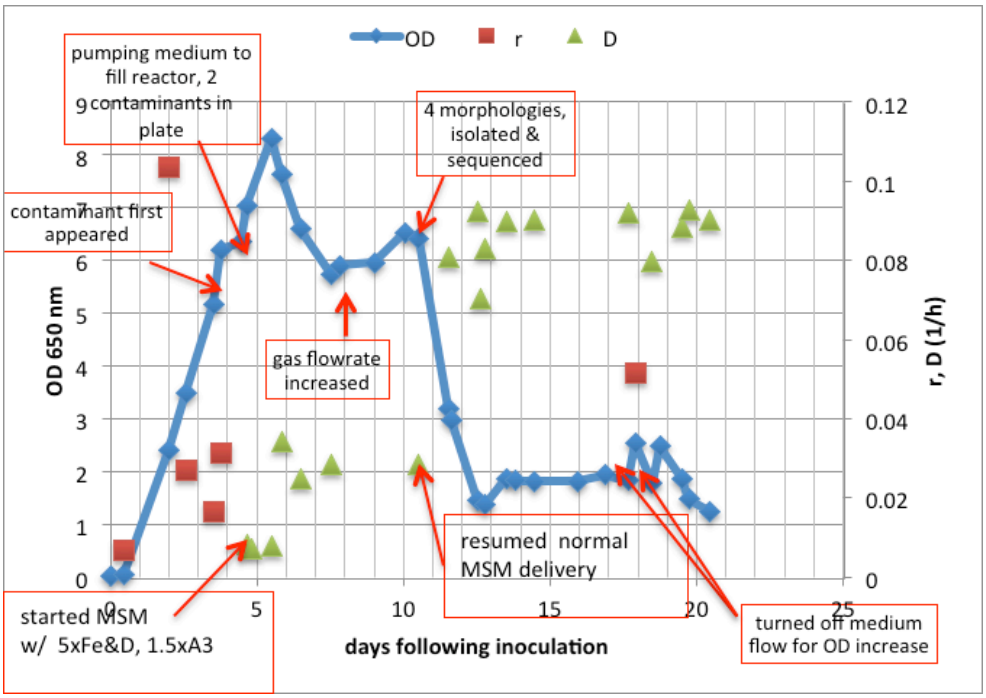
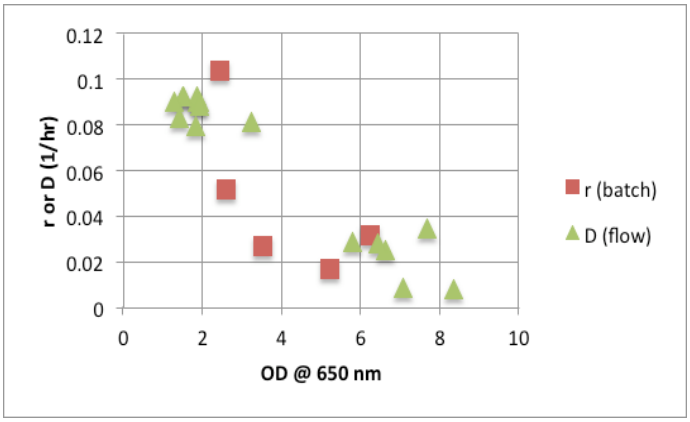


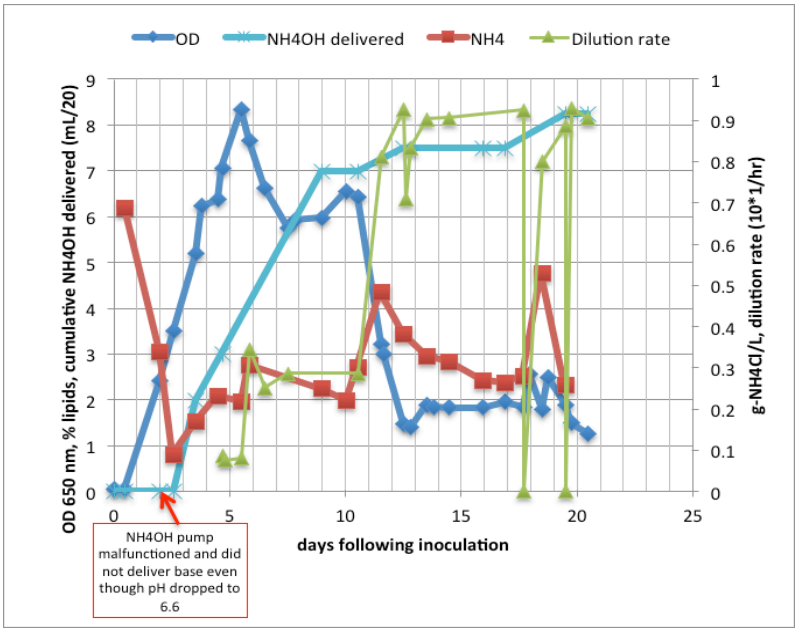
Figure 24: Correlation between Batch Phase Growth Rates (r) or Continuous Phase Dilution Rates (D) with OD



A modified input medium that had five times the normal amount of iron and trace minerals, and 1.5 times the amount of $MgSO_4$ was used for the 0.03 hr^{-1} dilution rate to continuously provide the required amendment addition without the need for any periodic nutrient addition as was required in continuous culture run #1. However, when the dilution rate was increased to

0.09 hr^{-1}, precipitation in the bioreactor was observed, and the use of this modified medium was discontinued. Consequently, the effects of this amended medium at higher growth rates were not tested. An estimation of nutrient requirements suggested that continuous supply of regular medium at the elevated dilution rate and reduced steady state OD, provided more than enough mineral supplementation of PO_4, Mg, Fe and trace minerals, such that incremental supplementation was not needed. At the high dilution rates, the rate of NH_4OH delivery for pH control decreased significantly, probably due to the increased supply of buffer with fresh replacement medium flow. During continuous flow, NH_4Cl concentrations ranged from 0.2 to 0.5 g-NH_4Cl/L, with the majority of NH_4^+ supplied by the replacement medium and not by NH_4OH provided for pH control. Figure 25 shows a plot of the NH_4^+ concentration over time along with the cumulative amount of NH_4OH added to the system for pH control, the measured OD, and the dilution rate (D). The initial drop in NH_4^+ concentration was due to a base pump malfunction. Once continuous medium flow began, NH_4Cl concentrations remained above 0.22 g/L. Peaks in NH_4^+ during this time correspond to increasing medium flowrates. Additional nutrients were added incrementally according to batch protocol up until the point of continuous flow at 4.6 days following inoculation.

Figure 25: Continuous Culture Run #2 NH_4+ Delivery and Concentrations



Gas composition, measured in influent gas and reactor headspace samples by GC, was on average 5.5% oxygen, 10% CO_2, 65% hydrogen, with the remainder nitrogen (Figure 26). Influent CO_2 ranged from 9.5 to 11%, and headspace values were equal to, or slightly lower than, the influent values. Oxygen influent values ranged from 5.0 to 5.8%, with headspace values lower by as much as 1.1% at the peak value of OD. Percent hydrogen in the influent ranged from 63-66%. The deviation of headspace hydrogen concentrations from the influent was inconsistent, but the dip around eight days following inoculation may be in response to

nitrogen accumulation during gas recirculation. Figure 27 shows the gas flow rates (VVM, volume of gas per reactor working volume per minute) through the working volume over the course of the run.

Figure 26: Composition of the Influent and Reactor Headspace Gases Determined by GC

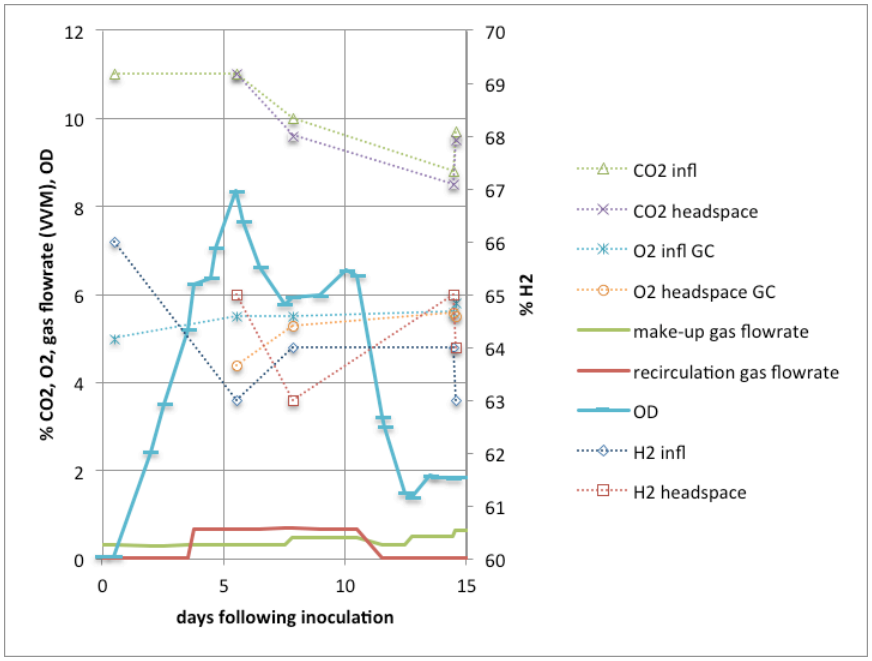


Figure 27: Continuous Culture Run #2 Gas Flow Rates

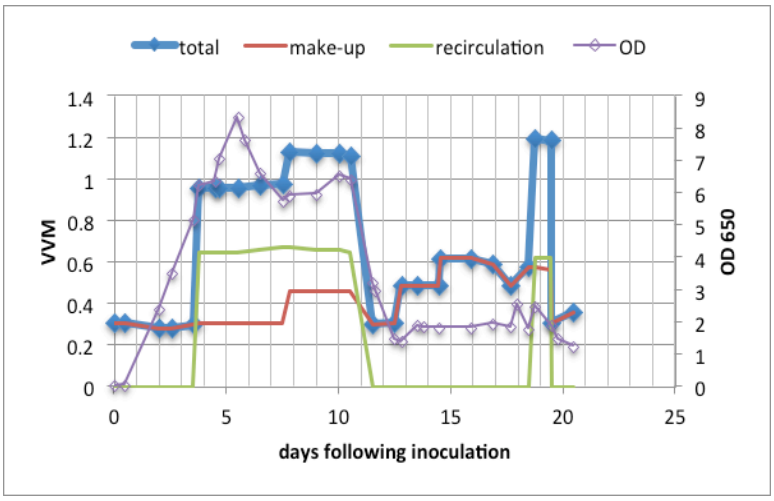


Figure 28 shows in-situ measurements of redox potential, DO and pH taken during continuous run #2. Over the first few days, the higher DO (~9%) corresponded to higher redox potential, and then both dropped. DO values fluctuated from ~1-2% through 5.5 days (the time of the

peak OD), and then remain relatively steady around 1%. It should be noted however, that the fiber-optic DO probe in the reactor became fouled with biofilm growth after several days, and therefore it is possible that the probe read DO at the base of the biofilm rather than capturing a representative value of the reactor broth.

Figure 28: Continuous Culture Run #2 Redox Potential, DO and pH

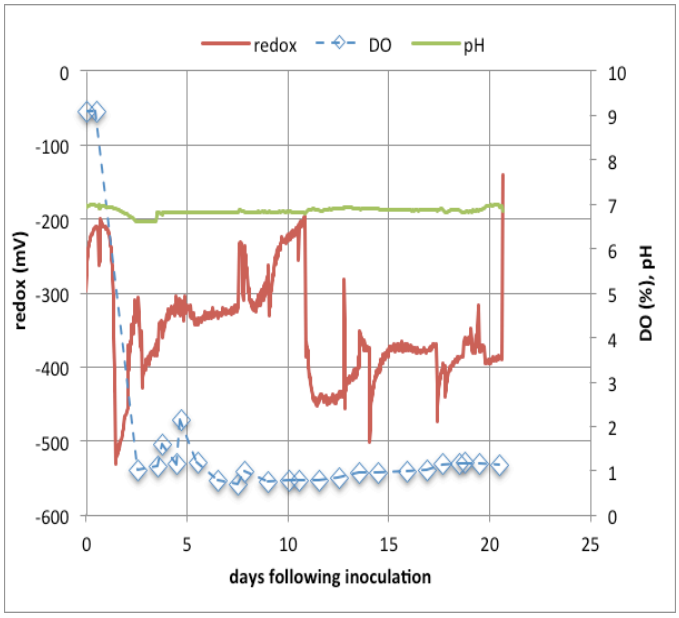


Table 4 shows the percentage by cell mass of lipid extract that is hexane soluble (non-polar or dipolar lipids), and extract that is chloroform/methanol soluble but hexane insoluble (polar lipids), at various sampling time points. Table 5 provides data from biomass harvesting where the accumulated outflow broth was centrifuged and the wet biomass pellets recovered from centrifugation weighed.

Table 4: Lipid Analysis of Reactor Samples from Continuous Culture Run #2

ID	Days post inoc	D (hr^{-1})	Sample mass (mg)	Chloroform/ methanol soluble (% of dry-cell mass)	Hexane soluble (% of dry cell mass)	OD
T06	4.65	0.009	39.7	14.36	0.76	7.06
T07	5.49	0.009	47.6	11.97	2.94	8.35
T08	6.49	0.03	59.3	6.91	2.19	6.62
T09	7.51	0.03	59.3	9.61	2.70	5.77
T11	10.03	0.03	59.7	6.03	1.68	6.55
T12	10.51	0.03	55.9	5.72	3.40	6.43
T13	11.52	0.08	36.3	6.34	3.31	3.22
average (excl T6 & 13)				8.05	2.58	6.74
Std Dev				2.67	0.67	0.96

Table 5: Biomass Harvest Data

Day of harvest post inoc	Volume of broth collected (L)	Wet biomass (g)	NH_4Cl (g/L)	OD	Average wet biomass productivity (g/day)
6.65	4.5	101.54	0.21	8.92	15
10.63	11.9	115.97	0.21	6.45	29
12.49	14.6	147.96	0.37	3.69	80
14.57	17	145.91	0.35	1.7	70
16.70	20	234.56	not sampled	1.9	110
18.49	16	143.31	0.25	2.09	80
20.07	21	284.22	not sampled	1.8	180

Following drying of the wet cell mass, the correlation between the dry cell mass density and the OD was determined. Based on the correlation, when the dilution rate was kept at a D=0.03 hr^{-1} and OD~6.6, a steady state dry cell weight of 3.1 g/liter was maintained. When the dilution rate was kept at a D=0.09 hr^{-1} and OD 1.8-2, a steady-state dry cell weight of 1.1-1.2 g/liter was maintained.

Some contamination was observed in the second continuous run and was first detected on the fourth day of operation, during the initial batch growth period. Contaminants were identified as species of the *Pseudomonas* and *Microbacterium* genera from isolates of samples taken on the 10th day. Additional contaminants isolated from the wall growth sampled from the bioreactor during harvest contained additional species of the *Achromobacter* and *Rhizobium* genera. While the quantitative fraction of each contaminant in the community was not determined, *C. necator*

was qualitatively seen to be the major constituent throughout the run based on plate streaking results and microscopic examination of culture sample. In subsequent testing, none of the isolated contaminants was able to grow chemolithoautotrophically on the $H_2/CO_2/O_2$ gas mix and MSM, and thus they most likely subsisted in the bioreactor system on organic byproducts of the *C. necato*r autotrophic metabolism.

3.4 Conclusions

Kiverdi demonstrated cell growth of the knallgas strain *C. necator* on a carbon dioxide and hydrogen gas mix under controlled conditions in a continuous bioprocess, successfully converting from batch operation. The continuous bioreactor system produced biomass under differing dilution rates and gas flows and media conditions. Two continuous flow runs operating in turbidostat mode were performed, each lasting 24 and 21 days, including the initial batch growth period of 3.7 and 4.5 days, respectively, which were needed to reach operating biomass density. During the period of continuous medium delivery, the reactor broth was continuously withdrawn, chilled to 4°C, and stored. Harvest of the collected biomass was performed by centrifuging the broth and recovering the wet biomass pellets. A total of 1.2 kg of wet biomass was harvested from a total of 114 L of medium and 1160 moles of H_2. Lipids from the wet biomass were extracted at the LBNL Advanced Bioprocessing and Demonstration Unit (ABPDU). The relatively low yield of biomass per H_2 fed into the system reflects the high proportion of H_2 that passed through the system unreacted, and points to the necessity of implementing a more thorough and complete recirculation of unconsumed gases in the bioreactor system, as well as increasing the gas conversion per pass through the bioreactor working volume.

It was found that the exponential growth rates attained in small batch runs could not be matched in the continuous culture runs. Factors limiting growth in the continuous runs appeared to be poorer delivery of oxygen to the culture due to inferior mixing/mass-transfer conditions at the larger scale. A deficit or excess of mineral nutrients may have played a role as well. It is possible that the use of different reactor architectures could improve the growth performance. The bioreactor used in the continuous system was chosen for its larger volume. The team's experience with somewhat smaller bioreactors (3.2 L) from a different manufacturer (Fairmentec), which were capable of higher-speed agitation (i.e. RPMs), as well as having a better mixing geometry, suggests that growth kinetics could be improved at the 5 L scale. Comparison of performance for increased agitation rates and different geometries of bioreactor should be tested in the future. Increasing productivity and gas conversion per pass can be accomplished by improving mass transfer of the low solubility H_2 and O_2 into solution through a variety of means including reducing bubble size, increasing liquid agitation and entrainment of bubbles within the working volume, increasing gas pressure, and increasing the water column height of the working volume. The various approaches to improving gas mass transfer will be discussed further in subsequent chapters.

There remains a need to further optimize the process for greater oil productivity. A major step towards increasing oil productivity is to switch to a knallgas strain that naturally accumulates higher lipid content, such as strains from the *Rhodococcus* genus, or to genetically modify *C.*

necator to produce higher quantities of the desired lipids. Other areas where the continuous process and set-up needs further improvement include:

- Improving gas utilization and reducing gas losses.
- Improving gas mass transfer into solution.
- Implementing the capability to run multiple turbidostats on gas in parallel.
- Optimizing mineral nutrient media concentrations for improved lipid yields and/or productivity.

Towards the end of improving the process in these various areas, the following is a list of some possible next steps that can be taken based on the experience with the continuous run experiments and set-up:

- Utilize continuous flow operation and set-up for further growth optimization.
- Implement smaller reactors to conserve medium and gas. For example, the collaborators at LBNL have a 2-liter bioreactor with the same internal agitation configuration as the 4.7 L bioreactors used in these experiment, and a range of working volumes from 0.4 to 1.6 L.
- Perform additional experiments to see if there is a correlation between dilution rate and lipid content.
- Increase the capacity of the gas recirculation component and closely monitor gas composition.
- Improve tubing connections to reduce gas leaks.
- Improve calibration of all flow and monitoring devices.
- Analyze nutrient system for optimization.
- Improve mixing by replacing the PEEK headplate and agitator with Fermac's stainless-steel version that can sustain speeds of up to 1000 RPM.
- Vary agitation rate and type and blade type – e.g., perhaps a vortex is necessary to get ideal synergy between reactor medium and gas bubbles.
- Reduce the risk of contamination by:
    - Increasing autoclave times.
    - Avoiding post-autoclave manipulations of the reactor and connections.
    - Minimize tubing external to the reactor, and eliminate recycle tubing.
    - Reconfiguring gas recirculation loop so that all components are autoclavable (consider using peristaltic pumps) or contained within sterile filter barriers.
    - Planning for a two-day operation of the reactor prior to inoculation to test for contamination.

CHAPTER 4:
Scale-up of Knallgas Microorganism Growth

4.1 Summary

The project culminated with scale-up of the oil production from CO_2, with confirmed production of fatty acid chain lengths in quantities that met the specifications and requirements of an industrial strategic partner customer. This work entailed scaling up the gas bioprocesses developed in the previous chapters. Larger scale (up to 20L) bioreactor runs on $H_2/CO_2/O_2$ gas mixes with *C. necator* were performed at SRI International, Menlo Park, California, and Iowa State University, in Ames, Iowa. The runs were based on the results of the bench-scale experiments described in Chapter 2. This chapter describes the laboratory facilities, the transition of the bench-scale protocol to the larger scale, and production results.

Oil samples totaling over 200 grams of oil were produced from CO_2 at the larger scale. The biomass from which the oil was extracted was fractionated through solute separations and recovery, and analyzed to identify biosynthetic products of the chemoautotrophic reaction. The lipids extracted from the knallgas microorganism cell mass produced an oil containing fatty acids having commercial applications as oleochemicals; as confirmed by a strategic industrial partner. Furthermore, the fatty acids can also be transesterified to produce biodiesel, thereby extending the application of the technology.

The scale-up work helped determine operational parameters necessary to initiate engineering and design on an integrated and continuous skid-mounted pilot plant (described in Chapter 5). Using the results of the experiments, a design was created for a pilot scale process to convert CO_2 into oils using knallgas microbes. The testing and operation of this pilot-plant would in turn enable scaling the reaction to a commercial production process for the conversion of CO_2 using knallgas microbes.

4.2 Larger-Scale (20 L) Batch Runs of *C. necator*

Kiverdi and SRI International performed batch cultivation runs of *C. necator* in 20 L bioreactors to produce several kilograms of biomass from CO_2.

4.2.1 Equipment and Experimental Set-up

Equipment and supplies were procured to grow knallgas organisms in 20 L bioreactors in an aqueous medium containing inorganic salts and buffers and gas mixtures of H_2, CO_2, and air. SRI installed two 20 L reactors (16 L working volume) from Applikon Biotechnology (Applikon) and purchased a combination of used and new parts to fully outfit the reactors with controls and sensors. A schematic diagram of the bioreactors and supporting systems is shown in Figure 29. The bioreactors were located in a fume hood to contain releases of hydrogen gas (Figure 30). All of the controls and gas sources were located outside of the fume hood to minimize exposure to hydrogen in case of a leak (Figure 31).

Figure 29: Schematic for Two 20-Liter Bioreactor System

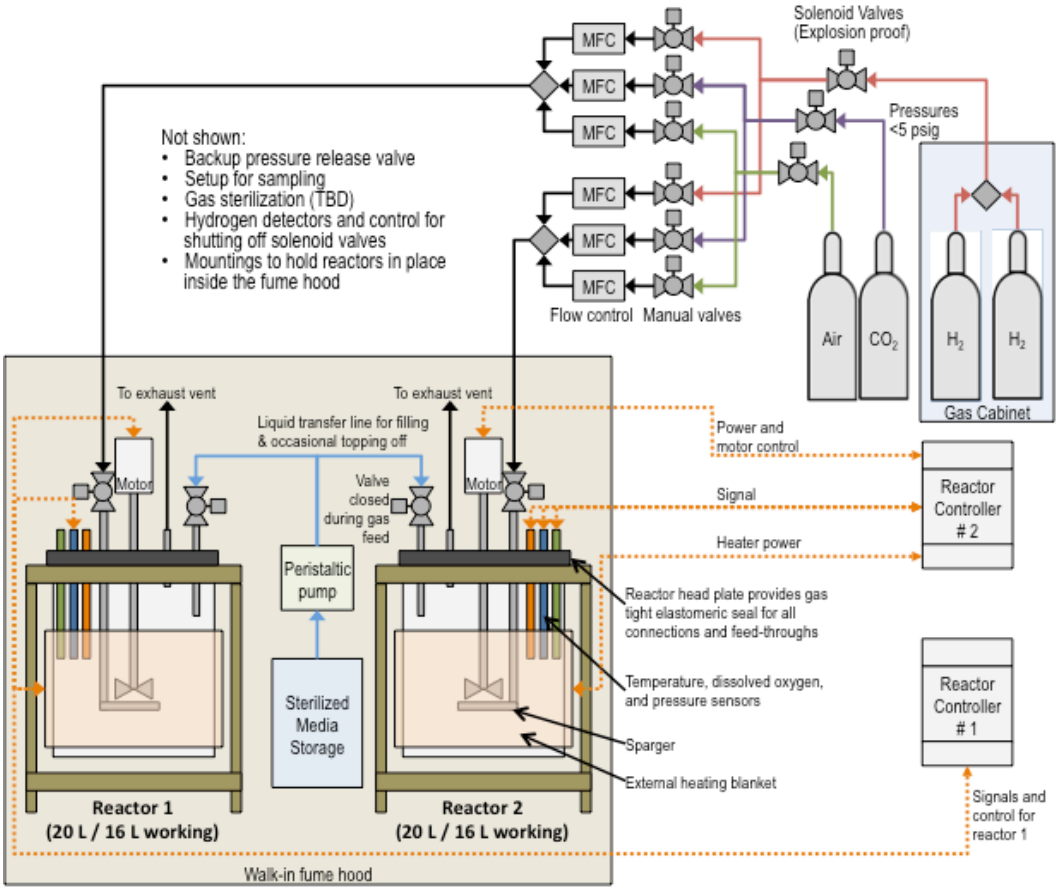
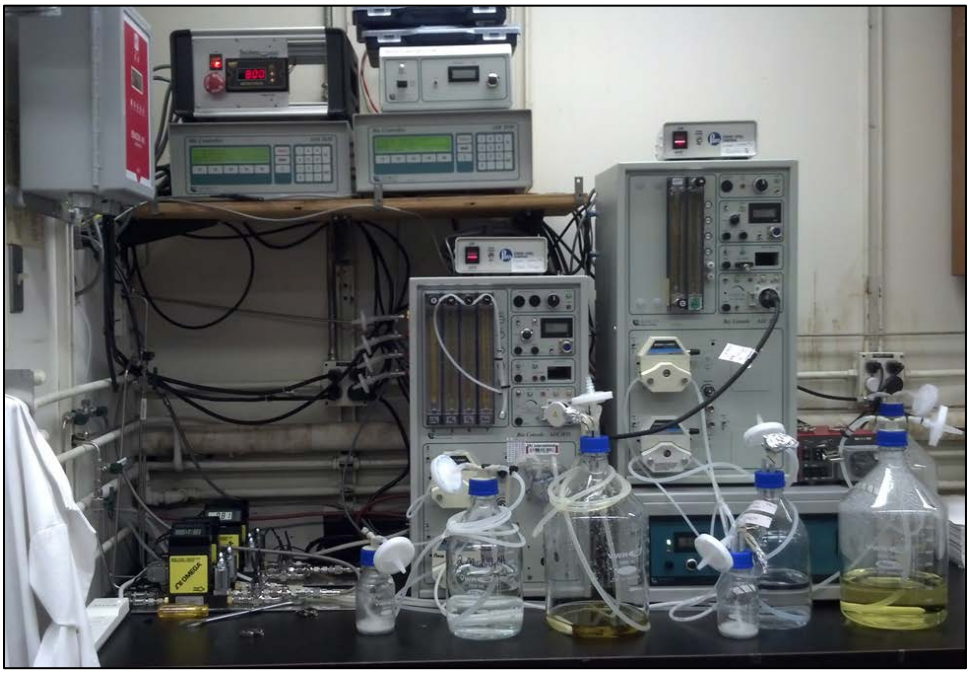


Figure 30: Installation of Two 20 L Bioreactors in a Chemical Fume Hood

Figure 31: Control System Used to Operate the Bioreactors, Gas Delivery, and Medium Addition



Bioreactor vessel: Each bioreactor consisted of a glass vessel mounted on a support stand with a stainless steel head plate having an elastomeric seal. The head plate had ports for numerous feed-throughs, all of which had an elastomeric seal to prevent the leakage of gas into or out of the reactor. These feed-throughs allowed for thermowells, pH probes, dissolved oxygen probes, gas inlets, liquid inlets, gas outlets, liquid sampling ports, and other items to all be mounted on the head plate. The reactor itself was mounted on a metal tripod holder. Clamps or chains were used to attach this tripod to the strut mountings located inside of the hood to prevent the reactor from being knocked over. The whole tripod and reactor setup was placed in a shallow plastic container to provide secondary containment

Bioreactor Sensors: A Pt 100 temperature probe located in a thermowell was used to monitor the temperature and to allow for control of a heater. A pH probe was used to monitor the pH and, if needed, control the addition of higher or lower pH buffered solutions to the reactor. A foam sensor was used to control the addition of anti-foam. A dissolved oxygen probe was used measure the oxygen levels in the reactor liquid and could be used to control agitation or open/close the gas flow to the reactor. All of the sensors were powered by, controlled by, and provided inputs to the bioreactor controller/console.

Stirring: A stirrer passed through the head plate with a complete seal and magnetic coupling. The stirrer had an external motor that was a separate item that fit around the external portion of the stir shaft. The motor speed was controlled by an external controller that allowed for precise control of the rotational speeds.

Heating/Cooling: The reactor was heated by an external electric heating blanket, which used a closed-loop proportional-integral-derivative controller (PID) controlled by the temperature probe via the bioreactor system controller. To maintain temperatures, a cooling finger was also plumbed to prevent overheating of the medium by the stirrer motor.

Controller/Console: The bioreactor system controller/console contained the components that controlled and operated the bioreactor system. These units provided the power, temperature control, stirring control, received inputs from the sensors, turned on and off the feed pumps (acid, base, anti-foam, and additional nutrients) based on sensor inputs, and were used to turn on/off the gas flows with solenoid valves and rotameters. Due to the lack of all stainless steel components, these units were not used to control the hydrogen to minimize the risk of hydrogen leaks. The controller/console units were located outside of the hood away from the bioreactors to minimize exposure to hydrogen in case of a leak and to minimize the time operators spend working directly around the bioreactors.

Figure 4.3 shows the Applikon controllers and consoles that were used to operate the reactors. These elements include controllers, consoles, stirrer controls, an explosive gas detection system, mass flow meters, level controllers, base control reservoirs, a medium addition reservoir, and a foam control reservoir. All of the reactor controls were located outside of the hood.

Gas Delivery: The gas was delivered into the lower portion of the reactor though a sparger setup that passed through the head plate. A valve located just outside the reactor enabled the gas flow to be manually shut off at each reactor separately. The gas feed line plumbed to the reactor was a flexible stainless steel line with a 0.2-micron filter installed at the reactor head to minimize possible contamination. Mass flow meters located outside of the hood were used to control the flow rates to the reactors. Lines between the cylinders and mass flow meters had both manual and solenoid valves for both manual and automatic shutoff of gases. The solenoid valves were connected to explosive gas sensors that would automatically shut off gas flows when hydrogen was detected in lab or in the hood.

Gas Storage: A gas cabinet was used to store the hydrogen cylinders. The gas cabinet was mounted in place and included ventilation and sprinklers. The cabinet included enough room to store multiple cylinders, and to allow for easy switching from an expended to a full cylinder.

Safety Controls: Gas detectors were used to determine the presence of hydrogen in the lab. Multiple sensors were located in strategic positions around the lab and in the hood. These gas detectors were configured to shut off the solenoid valves on the gas delivery lines if hydrogen was detected, resulting in shutting off the flow of gas to the reactors.

Peristaltic Pump: An additional peristaltic pump was located in the hood. This pump was used to transfer fresh medium into the reactors at the start of a batch run and used to remove the medium and biomass at the end of a batch run.

Medium Storage: Plastic carboys or glass bottles were used to store the fresh medium and the biomass recovered after a batch run.

Centrifuge: A Beckman Coulter Allegra X-12R centrifuge was used to centrifuge the broth harvested from a batch run to recover the biomass. The Allegra-12R provides refrigeration down to -10°C, is outfitted with a SX4750 swinging bucket rotor capable of 3,750 rpm and has a 3-L capacity.

4.2.2 Standard Operation of 20 L Bioreactors

4.2.1.1 Tech Transfer and Validation at 3 L scale

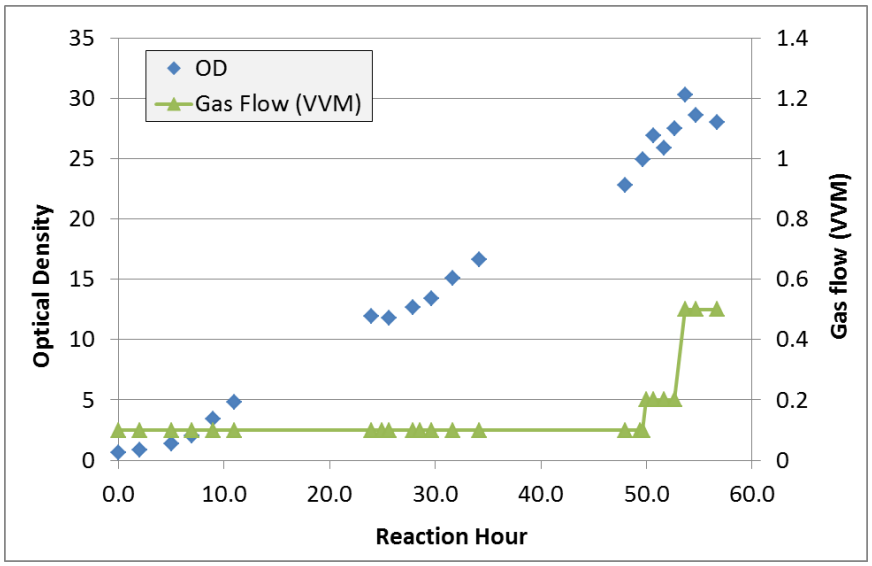
SRI first performed experimental runs at the 3 L scale in order to transfer technology from Kiverdi and to become accustomed to working with the organisms and protocols. Three runs were performed sequentially to establish and refine operational procedures for running at the 20 L scale (Table 6) which included stirring; heating/cooling; gas storage and delivery; safety controls; and medium recipe, preparation, and storage. The first run was inoculated with *C. necator* grown on gas in serum bottles and the subsequent two batches were inoculated with material from the previous batch. In each case, there was minimal lag time at the start of each batch, which demonstrated the ability of the organism to begin growth rapidly once gas is supplied, even if it is not in an active growth phase.

Table 6: Results from Preliminary Experimental Runs Performed in 3 L Bioreactors

Run #	Start OD	Final OD	Duration (Hrs)	Total gas flow range (VVM)	Stirring rate (rpm)	Inoculum
1	0.63	28	57.7	0.1 - 0.5	850	240 ml from serum bottles
2	2.96	32.4	103	0.2 - 1	900	~200 ml of run #1
3	5	40	99.9	0.2 - 1	1000-1200	~200 ml of run #2

The growth profile of a typical 3 L run is shown in Figure 32, which shows the optical density (OD) and total gas flow in VVM. An exponential growth phase can be seen at the lower ODs while above ODs of ~3, the growth rate is linear with a partial dependence on gas flow, suggesting the growth limiting factor is one of the gas components. This result may be due to poor mass transfer of the gases into the solution even at the higher gas flow rates, or another factor.

Figure 32: Batch Growth of C. necator at 3 L Scale



These preliminary runs established the following for application to the scale up runs:

- Procedures for heater control by the bioreactor controllers, although it was demonstrated that active cooling may also be necessary at larger scales.
- Determination of upstream gas pressures required for the mass flow controllers.
- Sterilization procedures for nutrient delivery.
- Baseline growth rates of the organism.
- Shear insensitivity of the organism and its ability to handle higher stirring rates.
- A method for monitoring of clogging of the sintered metal spargers by the organism after reaching higher ODs, particularly if there was low gas flow through the sparger.

4.2.1.2 Operation at 20 L Scale

Medium Preparation and Addition: The main medium component (A[28]) was prepared in 20-liter Nalgene carboys outfitted with sterile liquid transfer cap and filter assemblies. The medium was autoclaved in the carboys and transferred into the autoclaved reactors using sterile tubing and peristaltic pumps to avoid contamination. The smaller medium components (B[29] and D[30]) were prepared in large reservoirs and were sterilized by syringing the solutions through a single-use, sterile 0.2-micron filter directly into the reactor via the septum port. A fourth smaller medium component (C[31]) was handled in a manner similar to A, in that a larger reservoir outfitted with a sterile transfer cap was prepared with medium, autoclaved, and the medium was transferred using sterile tubing and a peristaltic pump.

Bioreactor Preparation and Start-up: Prior to each batch, the bioreactor was prepared for autoclaving. The reactor head plate was mounted in place. Transfer lines were connected,

clamped, and the end was covered with foil and sealed with autoclave tape. A 0.2-micron filter was connected to the gas inlet of the sparger to sterilize the incoming gases. A vent line was clamped and sealed with foil. The thermowell, condenser, foam level probe, cooling coil, sampling apparatus, adjustable liquid draw tube, and dissolved oxygen probe were installed. The port for the pH probe was covered and sealed with foil. The reactor was then autoclaved for 60 minutes at 131°C with a dry cycle. No medium was inside the 20-liter reactors during autoclaving due to the fact that the reactors had to be lifted and placed on their sides during autoclaving. The pH probe was sterilized with a combination of quick flaming, ethanol, and UV light. After the bioreactor was autoclaved and cooled to room temperature, the pH probe was inserted into the reactor while both the reactor and probe were inside a biosafety cabinet. The reactor was then mounted in the hood; i.e. cooling lines, transfer lines, electronic controls, heater, stirring motor, etc. were all connected. As quickly as possible, medium component A was transferred into the reactor to minimize the amount of time that the pH probe was dry. The temperature control and stirring were turned on, and if necessary, the cooling water as well. Once the temperature of the medium reached the desired temperature, medium components B, C, and D were transferred into the reactor via the methods described above. The pH control was then started.

Inoculum from Seed Culture: Fresh inoculum was prepared in serum bottles, if it was grown on gas, or in Erlenmeyer flasks if it was grown on sugars. The inoculum was usually provided in multiple containers, which were combined inside of a biosafety cabinet into a single sterile medium bottle outfitted with a sterile transfer cap assembly. An OD and streak of the inoculum was taken. The inoculum was then transferred into the reactor using sterile transfer tubing and a peristaltic pump. After inoculating the reactor, a starting OD of the batch was taken using the sample assembly.

Inoculating with the Previous Batch: The medium and biomass from the previous batch was removed via peristaltic pump except for the volume used to inoculate the next batch, which was typically < 1 liter. After removal of the material, sterile medium component A at room temperature was transferred in and the heating was turned on. The rest of the medium components B, C, and D were transferred in via the methods described above. Then the gas flow was turned on, stirring turned up, and pH control turned on. At this point, the run was considered to have started and a starting OD was taken. After the reactor reached the operational temperature the cooling was turned on.

Gas Composition And Flow Rates: The gas compositions used were those specified by Kiverdi. The ratios were controlled using mass flow controllers. The gas flow rates ranged from 0.05 to 0.3 VVM of total gas flow. Typical gas flow rates were 0.05 VVM over the weekends, and 0.2 VVM during the week when the operation of both reactors was under observation, and foam control, when needed, was provided through application of a chemical anti-foam agent. In the runs that did not use chemical anti-foam, typical gas flow values of 0.05 to 0.075 VVM were used to reduce the foaming to manageable levels.

pH Control: Ammonium hydroxide (2.0 M) was used to control the pH of the medium in the bioreactor. The ammonium hydroxide solution was prepared by autoclaving 1200 mL of MilliQ

water in a 2 L medium bottle outfitted with a sterile transfer cap and filter assembly and adding 800 mL of filter-sterilized 5.0 M ammonium hydroxide inside of a biosafety cabinet. The ammonium hydroxide was automatically transferred into the reactor via peristaltic pump, which was controlled by the bioreactor controller using the pH probe signal.

Nutrient Addition/Amendment: The nutrient amendment solutions used were the same as those used for the initial medium, with the amount of nutrient amendment added based on Kiverdi's growth protocol. The amendment solutions were either added directly into the reactor using a syringe and sterilizing through a 0.2-micron filter or added through sterile tubing that remained connected to the reactor using a peristaltic pump. In order to maintain stable mixing kinetics and prevent overflow, the total reactor volume was manually adjusted on a regular basis (typically daily) by removing small portions of the reactor medium and biomass to maintain a working volume of approximately 15.5 L. This was necessary to compensate for the water additions from the nutrient amendments and water generation by the cellular respiration.

Sampling: Small aliquots of the medium solution were taken at regular intervals from the bioreactor via the liquid sample assembly. These were used to perform the OD600 measurements on an Eppendorf Biophotometer Plus, as well as to provide the microfuged samples for DNA analysis. The microfuged samples were spun at 10000 rpm for 10 min, decanted, and stored at -20°C.

Foam Control: After reaching an OD of approximately 15, foam would start to fill the headspace and, if not controlled, the foam would easily fill up the 2 liter overflow reservoir overnight when gas flow rates of 0.2 VVM were used. A foam sensor was used to determine the presence of foam and to turn on a pump that would deliver a solution of silicon-based antifoam emulsion. Gas flow rates and stirrer speeds were adjusted as necessary in batches 11 and 12 to prevent excessive foam build-up. At gas flow rates of 0.05 VVM to 0.075 VVM, the bioreactors were able to be operated without anti-foam. However, the foam would fill the headspace; causing a small amount to flow into the foam overflow container via the gas outlet.

Biomass Storage and Centrifuging: After a batch, the biomass and medium were transferred out of a reactor using a peristaltic pump into 10-liter polypropylene jerry cans. The jerry cans of biomass and medium were stored in a refrigerator until they were centrifuged. The biomass was centrifuged 3 liters at a time, split between four 750-mL polycarbonate centrifuge bottles. The centrifuge was operated 3,750 rpm at 4°C for 30 minutes. The supernatant was decanted off and sterilized with bleach prior to disposal. The biomass for a single batch was combined and stored in polypropylene bottles in a refrigerator until they were picked up by Kiverdi.

4.2.3 Summary of 20 L Batch Runs

A total of nine runs were performed with *C. necator* in 20 L reactors. Most of the runs achieved high cell densities between 39-50 OD (17-22 g/L) (Table 7) with the exceptions resulting from either lower gas flows (run #11) or low stirring rates (run #5). The highest cell density, (22 g/L) was achieved in run #7 in 168 h, corresponding to a batch productivity of 3.1 g biomass/L/d.

Table 7: Summary of 20 L Runs with *C. necator*

Run ID#	Scale	Reactor	Start OD	Final OD	Duration (Hrs)	Total gas flow range (VVM)	Stirring rate range (rpm)	Inoculum
4	20 L	A	0.94	42	248	0.1 - 0.3	600-800	~600 ml run #3 from 3L batches (Table 5.1)
5	20 L	B	1.1	6.7	188	0.05 - 0.2	200	~ 650 ml of run #4
6	20 L	A	0.085	42	165	0.05 - 0.2	800-900	~ 400 ml of sugar grown culture
7	20 L	B	1.2	50	168	0.05 - 0.2	200-850	~ 500 ml of run #6
8	20 L	A	2.1	42.5	167	0.05 - 0.2	800-980	<1 L of run #6
9	20 L	B	3.5	49.4	165	0.05 - 0.2	750-850	< 1 L of run #7
10	20 L	A	1.9	39.2	143	0.05 - 0.2	800-950	<1 L of run #8
12	20 L	B	0.56	37.3	264	0.05-0.1	500-750	<500 ml of run #10
13	20 L	A	2.34	29.2	143	0.04 - 0.2	800-850	~750 ml of run #12

For most of the batches, the 20 L reactors were inoculated on a Friday and run at reduced gas flow rates (0.05 VVM) over the weekend during the lag phase growth of the organism. Flow rates were limited to these low values over the weekend to prevent the need for replacing compressed hydrogen gas-cylinders during this time. For every batch, the temperature, pH, and OD was monitored and recorded. Samples were withdrawn at the beginning and end of each run and periodically throughout the run for analysis

The first 20 L run in reactor A (run #4) was inoculated with material from the last run (run #3) in the 3 L reactor (Table 6). During this batch, different gas flow rates and stirring rates were explored. It was established that active cooling was needed, particularly at higher stirring rates (>700 rpm). External fans were used to help cool the reactor and a cooling coil was installed for subsequent runs. By the end of this initial shakedown run at 20 L scale the OD had reached 42 over 248 h (Table 7).

The following run (run #5) was performed in reactor B, which had a stirrer motor and controller that was limited to 200 rpm. This was not enough agitation to create a sufficient vortex to bring headspace gases back into the reactor and contributed to this batch only achieving a final OD of 6.7. After installation of a new motor and a controller and a cooling coil, this reactor performed similarly to reactor A in subsequent runs.

Inoculum was grown on sugar to inoculate run #6. The organism appeared to switch easily to gas growth with the OD increasing by the end of the day and continuing to increase over the

rest of the run. The subsequent batches 7-10 were all grown from inoculums that originated from this batch via sub-culturing.

Runs #7-10, were all grown under similar conditions. For each batch, inoculation occurred on a Friday. A total gas flow rate of 0.05 VVM was applied over the weekend following inoculation, and then the total gas flow rate was increased on Monday morning to 0.2 VVM for the rest of the run. The stirring rates were also increased (to 800-950 rpm depending on impeller configuration) in order to increase the recycling of the headspace gases into the liquid to improve mass transfer. These runs achieved final ODs between 39 and 50. Runs 7 and 9 that were run in reactor B had higher final ODs compared to the runs in reactor A, which may be attributed to a larger upper impeller in reactor B that would have led to improved vortex mixing and thus improved recycling of gases from the headspace even at lower stirring rates. After these runs, the upper impeller in reactor A was replaced with a larger one. Heavy foaming was encountered during these runs and the automatic addition of anti-foam was utilized to reduce the foaming.

For the final runs (#12 and #13), conditions were used to avoid the use of anti-foam by reducing the flow rates and stirring rates. Throughout the runs, the gas flow rates were decreased as needed to avoid the over-foaming. The lower gas flow rates (0.05 VVM), particularly at night, resulted in lower growth rates than previous runs. During the day, when personnel were present to observe the bioreactor, and make adjustments as needed, the reactor was operated with slightly increased gas flow rates of (0.075 VVM). At higher ODs, the foaming became more extensive and thus reduced gas flow rates had to be used compared to the beginning of the batches. After observing the runs for a couple days and adjusting both gas flow rates and stirring rates, it was realized that the gas flow input into the reactor had a larger impact on foaming than the stirring. This eventually led to operating with high stirring rates and lower gas flow rates. These runs achieved ODs of 29 and 37 with no anti-foam used.

General observations and comments from these runs are included in Appendix A.

At the end of every run, the medium and biomass were transferred out of the reactors into jerry cans using peristaltic pumps. The material was stored in a refrigerator, and later was centrifuged 3-L at a time and stored in a refrigerator until used for extraction of the oil.

Monitoring of culture purity was performed by morphological assessment on nutrient agar and microscopic observations. Samples of inoculum were plated at the onset of several runs and then again at the completion to confirm culture purity and monitor the emergence of any contamination. Approximately 10 uL were plated onto the agar plates and incubated at 28°C for at least four days. After incubation, the plates were examined visually and under a dissecting microscope to determine colony homogeneity/purity and determine the presence/absence of any contaminant. Some of the cultures were also examined microscopically (oil immersion 100x/10x) for homogeneity. Under the bioreactor conditions used, *C. necator* consisted of very dense cultures of mostly single short rods (~1 um x 1.5 um). Some doublets were present, and some were highly mobile, with no clumping. Colonies on agar were off-white, glistening and somewhat mucoid, denser/darker in the center, convex, and ranged from entire to slightly

undulate. In some of the batches, a fungal contaminant was detected early in the run that suggested it arose during inoculation. However this fungal contaminant did not appear to effect the growth of *C. necator* during the bioreactor run.

4.2.4 Lessons Learned

Foam Production: At ODs more than 15, the broth in the bioreactor would begin to foam. This foam was capable of filling the headspace and leaving through the exhaust line. A silicone based anti-foam agent worked well to easily remove the foam. In the two runs at the 20 L scale that were run without anti-foam (runs 12 and 13), the gas flow rates and stirring rates were adjusted to manage the foam and keep it from overflowing from the reactor. Through trial and error of adjusting the two parameters, it appears that the foam issues of overflowing were due primarily to gas inlet flows rather than stirring rates. It was found that it was possible to successfully operate the reactors with high stirring rates (>800 rpm) and reduced flow rates (<0.05 VVM) and not have the foam leave the reactor. However, this lower gas flow rate resulted in slower growth rates. At larger scales, the bioreactors used to grow *C. necator* must be designed to handle or remove foam. In future experiments, the content of *C. necator* in the foam should be tested, since in some bioreactions the foam can have high concentrations of organisms, and thus significant yield could be lost if the foam is disposed of without harvesting the cell mass it contains.

Temperature Control: It was found that passive cooling through reactor walls was insufficient and thus active cooling with cooling water was needed. It was also found that higher stirring rates resulted in higher temperatures. This could be due to friction from the stirring the liquid, waste heat generated from the motor, and from increased metabolic heat generated by the microorganisms due to increased growth rate with agitation. It was found that *C. necator* can survive and recover from elevated temperatures of at least 35-37°C.

Shear Insensitivity: *C. necator* does not appear to be sensitive to shear stress and was able to handle high stirring rates in the range of 900-1200 rpm. The ability to operate at higher stirring rates improved both the breakup of bubbles and created a vortex that brought headspace gases back into the liquid that subsequently improved the growth rates.

Sparging /Gas Delivery: Clogging of the sintered metal frit sparger in the 3 L reactor occurred, which led to the inability to deliver gas. It was suspected that the clogging occurred when the gas flow to the reactor had to be shut off during the weekend. In the 20 L reactors, L shaped spargers were used that had small holes drilled on the bottom. These holes were much larger than the pores in the frit. There were a couple instances of clogging of these holes, even though the gas feed was never stopped. However, simply increasing the gas delivery pressure by a few psig overcame these clogs. At larger scales, it will be important to have a reactor design that allows for a method of removing/cleaning a sparger without requiring a complete shutdown of the reactor system. Also, a rubber/plastic sparger or membrane, versus the metal sparger used, could be tested in future experiments.

4.3 High Cell Density Runs at 15 L Scale

A collaborative effort was performed with researchers at Iowa State University (ISU) to further develop technology for the use of carbon dioxide (CO_2) for the production of chemicals and fuels. Match funding for this work, performed at ISU, which supported the project objectives and goals, was provided by a grant from the Iowa Economic Development Authority. Part of the effort was to develop the bioprocess for growing the strain *C. necator* at larger scale and high cell densities.

Seed cultures were prepared in 4X250 ml flasks for 36 hours and then inoculated into 15 L glass bioreactors with 5-7 L working volumes. A Bioflo 310 control unit (New Brunswick™ Eppendorf) was used for controlling temperature, pH, and dissolved oxygen (DO). Gases were supplied from a compressed H_2 tank, a compressed CO_2 tank, and compressed air, each set at 20 psi. H_2 and CO_2 were mixed and flowed through a single line for delivery into the bioreactor vessel, while air was delivered to the vessel through a separate line. A pressure gauge was used to monitor the gas delivery pressure to the bioreactor. During the batch run, medium pH was controlled in the range of 6.8-7.0. The medium pH was adjusted through 2N NH_4OH solution additions. Concentrated nutrient stock amendments B, C and D, as described in the previous section 4.2, were added during the batch run into the bioreactor based on Kiverdi's protocol. Antifoam C was used to control foaming when necessary.

During the batch runs, sub-culturing was used to shorten the turn-around time between runs and the lag phase of the batch culture. In brief, when OD reached a later exponential/linear phase, the majority of the culture broth was discharged for cell mass harvesting, while 100-200 ml broth was kept in the tank to be used as seed for the next batch culture. Thus, runs inoculated in this manner represented more of a semi-continuous process rather than a completely batch process. A similar approach was also used by SRI, as discussed in the previous section.

A total of seven batch runs were successfully completed with *C. necator* (Table 8), with a maximum cell density of 41 g/L achieved, corresponding to a biomass productivity of around 6.9 g/liter/day cell dry weight productivity.

Table 8: Summary of High Density *C. necator* Runs at 15 L Scale

Run ID	Final OD	Final volume	Cell density (g/L)	Total dry weight
1	19	5 L	8.2	41 g
3	51	5.5 L	23	126 g
3a	97	8.25 L	41	340 g**
4	20	6.5 L	8.5*	55.6 g**
A	15	6.5 L	6.3*	40.8 g**
B	49	6.5 L	20*	132 g**
B1	91	7 L	38*	267 g**
				1003 g

* The cell density for these batches (4, A, B, B1) was estimated based on ratio of OD vs dry weight (OD=0.42g/L) which is obtained from 3 and 3a batches.

** The dry weight is estimated from wet biomass weight.

The major problems encountered in the *C. necator* batch runs were foaming and high (wasted) hydrogen use due to the low conversion of H_2 per bioreactor pass and the lack of gas recirculation. Foaming problems became significant in the OD range of 10-30, and then stabilized and even decreased when the OD surpassed 50. Antifoam C was found to be ineffective. However, it was found that enriching the MSM and nutrient amendments with ferric iron helped to inhibit foaming, although excess addition of ferric can inhibit the cell growth. These results are the opposite of what was observed at SRI at 20 L scale where anti-foam C was found effective against foaming, while ferric supplementation was found to be ineffective, as discussed in the previous section. Further work is required to understand the effects of antifoam, type of antifoam, and ferric additions on foaming control. Also the discrepancy in the respective effects of anti-foam C and ferric supplementation observed at SRI and ISU needs to be investigated.

The Kiverdi-ISU collaboration succeeded in growing *C. necator* cultures at the 15 L scale to 41 g/L dry cell weight over the course of 5-6 day batch runs, corresponding to the highest productivities achieved in 1 L bioreactors at LBNL (see Chapter 2). Collectively, ~1 kg of *C. necator* dry biomass was produced. The *C. necator* biomass produced in these runs contained 2.3% (dry basis) of neutral lipid (e.g. TAGs) and ~50% PHB content.

4.4 Conclusions from Larger Scale Production

These larger-scale tests indicated the *C. necator* strain that could be grown to high cell density on CO_2 at a scale 5 to 10 times greater than had been done in earlier work. Under conditions used in these studies, this strain produces PHB as a principal cellular product and accumulates ~7% hexane extractable oil. Internal analysis and external validation by the project's strategic industrial partner determined this hexane extractable oil is primarily composed of a mixture of saponifiable lipids comprising TAGs and lecithin type lipids called phosphatidylcholines.

Phosphatidylcholine (PC) is a dipolar, hexane soluble phospholipid that is often a major component of biological membranes. Fatty acids are the primary building blocks of both these TAG and PC lipid molecules composing the oil, and can be recovered using standard hydrolysis techniques.

This work demonstrates CO_2 can be converted to mid- to long- chain fatty acids. It also demonstrates a process converting CO_2 into products using knallgas microbes can be developed and scaled. However, a practical commercial fatty acid production process using *C. necator* would likely require genetic modification of the strain to reduce the PHB content and increase production of the target molecules. Alternatively, *C. necator* could be replaced in the process with another knallgas strain having a greater natural propensity to oil production, such as one of the species of *Rhodococcus*. With relatively straightforward design changes, strain selection and modification, and process intensification, the yield from CO_2 can be increased, and a commercially viable system for converting CO_2 to targeted products can be built. In work beyond the scope of this project Kiverdi has been investigating both the avenue of genetically modifying *C. necator*, and that of implementing comparable knallgas bioprocesses with naturally oleaginous knallgas strains such as *Rhodococcus* species. In CHAPTER 6: Pathway Engineering to Create Novel Products, some of the subsequent work in genetically modifying *C. necator* will be described.

To summarize, work described in this chapter focused on performing larger scale production runs to demonstrate conversion of CO_2, and full-functionality of oil production from CO_2, at increased scale. This work supported and informed pilot scale reactor design, and proved out the integrity and basic operability and safety of the designs. The planning and execution of the production runs involved determining and targeting conversion efficiency of CO_2 to an oil that met a customer's specifications, performing the oil production runs, and shipping the oil product to the strategic industrial partner for testing and evaluation. The strategic industrial partner independently confirmed and validated that the oil contained fatty acids they currently use in oleochemical production, which could be recovered through standard hydrolysis methods typically applied to vegetable oils such as palm oil.

CHAPTER 5:
Pilot Plant Design

5.1 Summary

A pilot plant design for conversion of CO_2 into targeted products from using knallgas microbes was created. For *C. necator*, the natural products would be the polyester polymer PHB, amino acids, and/or single cell protein (SCP).

After a process for converting CO_2 to biomass using knallgas microorganisms had been implemented at the laboratory scale, Water Works Engineers was contracted to develop the following short-term scale-up and pilot plant designs:

1. Biomass production at 50 L bioreactor scale.
2. A mobile pilot plant containing 50 L scale production equipment to allow testing on CO_2-containing gases at various client sites.

The initial biomass production pilot facility was specified to have a 50 L reactor (which can be easily disinfected), temperature control for the reactor (primarily cooling, since the biological reaction is exothermic), active mixing, and to have the controlled feed of H_2, O_2 (or air) and CO_2 gasses, along with nutrients for the microbes and caustic solutions for pH control. The design assumes that this initial facility would run sequential batch processes for several months duration.

This chapter presents the details for a design of such a 50 L pilot plant, installed in a converted shipping container. A process flow diagram and layout of the plant in a shipping container are provided in Appendix B.

5.2 Gas Transfer Design Considerations

5.2.1 Gas Usage

The knallgas microorganisms metabolize H_2, CO_2, and O_2 to produce cell mass, and water as a byproduct. Estimates for gas utilization (Table 9) were generated based on lab scale experiments described in Chapter 2. Specifically, the microorganism *C. necator* is assumed to use 4 moles of H_2 per mole of CO_2 and 5 moles of H_2 per mole of O_2. Furthermore, the growth of 3 grams of cell mass on about 1 gram of H_2 gas was assumed, which is somewhat below the ~3.3-3.6 g/g H_2 measured experimentally at the bottle scale (2.4.2 H_2 Utilization Efficiency).

Table 9: Unit Gas Usage

Gas	Moles	Volume	MW	Gas Density (g/L)	Mass Fraction (g/L of gas)	Mass Fraction	Ratio (g gas/ g cell mass)
H2	1.00	69.0%	2	0.089	0.062	10.3%	0.333
O2	0.20	13.8%	32	1.429	0.197	33.0%	1.067
CO2	0.25	17.2%	44	1.964	0.339	56.7%	1.833
	1.45	100.0%			0.597	100.0%	

5.2.2 Single-Pass Gas Transfer

The lab-scale experiments were generally operated with a single-pass gas transfer; i.e., the mixed gas supply is added to the bioreactor vessel via bubbling or sparging, gas is diffused into solution, and the excess unconverted gas is removed from the head space of the bioreactor and vented off. The exceptions were the continuous runs described in the Chapter 3 where some recirculation of headspace gases was implemented. For the total gas supply target of 1.0 VVM (volume of gas/ bioreactor volume/min) and the 50 L pilot scale reactor, the gas supply for a single-pass approach would be 50 L/min. The target biomass concentration for the pilot bioreactor was a dry cell weight density of 30 g/L within three days, or a growth rate of 10 g/L/day. The gas delivery and conversion parameters when using a single - pass approach to achieve the necessary growth rate are shown in Table 10.

Table 10: 50 L Pilot Reactor Single-Pass Gas Supply for 10 g/L/d Biomass.

Gas	Grams gas per gram cell mass	Max Cell Use (g/L/d)	Max Cell Use (mg/L/hr)	Max Cell Use (g/d)	Mass Fraction (g/L of gas)	Gas Supply at 1 vvm (g/d)	Apparent Gas Conversion
H2	0.333	3.33	138.9	166.7	0.062	4,433	3.8%
O2	1.067	10.67	444.4	533.3	0.197	14,187	3.1%
CO2	1.833	18.33	763.9	916.7	0.339	24,384	3.1%

The predicted gas transfer efficiencies of 3.1-3.8% is very low, as one would expect with single - pass gas diffusion through a relatively shallow reactor. Typical oxygen transfer conversion for fine-bubble diffusers are about 1.5 to 2.0% per foot of submergence[32]. The 50 L pilot would have a diffuser depth of about 24", and therefore the gas transfer efficiency for a single-pass gas diffusion system would be expected to be ~ 3-4%. With these transfer rates, for a 50 L vessel being fed a gas mix at 1 VVM, the gas flow rate would decrease from 50 L/min (inlet supply) to only 48.1 L/min (exhausted) from expected incorporation into biomass. Consequently >95% of gas is wasted if it is exhausted after a single pass, making gas recirculation necessary.

Particularly challenging is the very high oxygen transfer rate (dO/dt) required: 444 mg/L/hr). A brief description of the mass transfer of gas molecules into the liquid phase is as follows. The gas initially (and very quickly) transfers from the gas phase to the liquid side of the gas/liquid interface to its saturation pressure predicted by Henry's law. Saturation concentrations for gas mixtures considered here with a reactor operating at 1 atm pressure and at 20 °C are shown in Table 11.

Table 11: Gas Saturation Concentrations

Gas	Volume	Partial Pressure (atm)	K_H (mole/atm/L)	moles/L	MW	mg/L
H2	69.0%	0.690	7.80E-04	5.38E-04	2	1.1
O2	13.8%	0.138	1.80E-03	2.48E-04	32	7.9
CO2	17.2%	0.172	3.40E-02	5.86E-03	44	257.9

The difference between the saturation concentration (C*) occurring at the gas-liquid interface and the average bulk liquid concentration (C_L), is the driving force for dissolution and diffusion of the gas molecules into the bulk liquid solution, given by the following equation:

$$\frac{dC}{dt} = (K_L a)_f (C^*_{\infty,f} - C_L)$$

The gas transfer coefficient (K_La) represents the efficiency of the gas transfer system in translating a concentration gradient of dissolved gases into a flux of gas molecules through the liquid phase. Since the reaction of H_2 and O_2 catalyzed by the knallgas metabolism is extremely energetic, it was assumed the concentration of hydrogen dissolved in the bulk solution is approximately reduced to zero. With this assumption, it can solved for a K_La for H_2 needed to meet the metabolic demands for the targeted productivity of 10 g/l/d. The result is shown in Table 12. The K_La for O_2 and CO_2 is estimated by rescaling relative to the K_La for H_2, accounting for the fact that diffusivity of these gases in water is less half that of H_2. Given these estimated K_La values and the known equilibrium concentrations of the gases by Henry's law, the bulk liquid concentrations of the O_2 and CO_2 that will deliver the flux of these respective gases required by the knallgas microorganisms metabolism can be calculated. This model predicts that given a concentration of H_2 in the bulk liquid of roughly zero (our assumption), the bulk liquid concentration of dissolved oxygen would be reduced down to about 0.6 mg/L, and the dissolved CO_2 to just over 240 mg/L, by the metabolic demands of knallgas microorganisms producing biomass at a rate of 10 g/l/d.

Table 12: Single-Pass Gas Transfer

Gas	Diffusion of Gas in Water	k_La (1/hr)	Use (mg/L/hr)	Liquid Conc. (mg/L)	Gas Mass Transferred (g/min)	Headspace Gas (g/min)	Headspace Volume Fraction
H2	4.5	129.1	138.9	0.0000	0.116	2.963	69.0%
O2	2.1	60.2	444.4	0.5675	0.370	9.482	13.8%
CO2	1.92	55.1	763.9	244.06	0.637	16.297	17.2%

It should be noted that the K_La estimated for oxygen, 60/h, is a relatively high number compared to what is commonly attained in waste water treatment and aerobic bioprocess fields[33, 34], and therefore, may be challenging to achieve in conventional bioreactor designs. If the K_La requirements are not met, then gas transfer would become the limiting step in the production of biomass.

5.2.3 Gas Transfer with Headspace Gas Recirculation

Because of the high volume of gas effluent associated with single pass gas transfer, the pilot plant is designed with a gas recirculation loop as a means of increasing the overall gas efficiency. In this configuration, a gas recirculation pump draws gas from the bioreactor headspace, which is blended with fresh mixed gas from a gas make-up supply, and the blended gas is delivered to the gas diffusion stones in the reactor. The gas recirculation loop is sized such that the blend gas of recirculated and fresh make-up gas will pass through the working volume at a rate of 1 VVM, or a gas flow rate of 50 L/min, to match the rates for achieving fast growth and high cell densities at the lab scale.

As discussed, less than 4% of the gas supplied to the reactor per pass is expected to be incorporated into biomass. Consequently, of the gas supplied into the working volume, only around 4% of the total gas (about 2 L/min) will be make-up gas drawn for the fresh gas supply.

5.3 Pilot Plant Description

5.3.1 Key Equipment and Process Design

Liquid Recirculation

A constant-speed progressive cavity pump is provided to recirculate the culture broth. The primary purpose of the liquid recirculation pump is to allow for chemical addition into, and monitoring of, the culture broth outside of the bioreactor environment. This liquid recirculation pump also induces mixing in the bioreactor vessel. The liquid recirculation pump runs continuously while the bioreactor is in operation, drawing from the bottom of the bioreactor vessel and discharging at the top, resulting in the liquid flow counter-current to the gas flow. The pump is also connected to a clean-in-place (CIP) tank to allow for addition of cleaning chemicals to the circulation loop and the bioreactor. The CIP tank may also be used for initial batching of basal medium into the bioreactor.

A 0.2 micron sanitizing filter is provided in a bypass loop of the recirculation piping to allow for sanitizing of the basal medium and other liquids during initial startup of the reactor. Both pH and oxidation/reduction probes (ORP) monitor the conditions in the recirculating fluid. The pH value can provide feedback to one of the chemical feed pumps (initially NH_4OH, later NaOH) to control dosage used to maintain a constant pH. The liquid recirculation pump can also be used to transfer bioreactor contents to the centrifuge.

Bioreactor Temperature Control

The bioreactor is supplied with a water jacket to maintain the bioreactor contents at a constant temperature. An external heating/cooling recirculating chiller provides cooling and heating capacity via an internal pump to circulate the cooling fluid.

Gas Supply

As described above, the fermenter is fed with H_2, O_2 and CO_2. O_2 and H_2 gas are stored in small cylinders ("T" size for H_2 and "K" size for O_2, which give maximum storage volumes of 1.6 lbs. (280 standard cubic feet (scf)) of H_2 and 20 lbs. (230 scf) for O_2) outside of the pilot container. The small cylinder sizes keep the installation within fire code, and avoid additional facility siting requirements. These gas cylinders are changed out prior to each batch cycle. The CO_2 gas cylinder should be at least 10 pounds, but a larger cylinder may be used without having a negative impact on the fire code ratings.

Each of the cylinders store gas at approximately 2200-2400 psig. A pressure reducing valve on each cylinder reduces the pressure of the outlet gas to approximately 175 psig. Flow rates of each gas are measured using a rotameter specific to each gas so that density calibrations are unnecessary. A 3-gas proportioner is used to adjust the mix of the gas to match the stoichiometric requirements. The flow rate of the mixed gas is measured using a rotameter and filtered with a 0.2 micron membrane filter. The mixed gas is supplied to the bioreactor in response to the bacterial consumption, so the gas feed rates through the proportioner will be variable and very small during the initial growth phase of the organisms reaching a maximum towards the latter stage of the run. A mixed-gas tank (using a standard LPG tank) is provided to allow batch blending a large volume of gas, and then allowing the bioreactor to draw from the blended gas tank as necessary. The tank is sized to have sufficient storage volume for the needs of a complete bioprocess cycle. A low-pressure regulating valve is used to feed the mixed gas into the gas recirculation loop upstream of the gas recirculation pump. The gas is fed in direct proportion to the gas consumption in the bioreactor. Headspace gas is continuously recirculated from the bioreactor headspace through diffusers at the bottom of the bioreactor (which then bubbles through the working volume of the bioreactor, ending in the headspace). As gas is transferred into solution, the gas pressure in the headspace will slightly reduce below the pressure regulator setpoint (slightly positive to atmospheric pressure), causing the regulator to open and introduce fresh mixed gas into the recirculation loop.

Gas Recirculation

As described above, a gas recirculation loop is used to continuously provide gas transfer in the bioreactor. The gas recirculation pump is a UL-listed, explosion-proof (Class I, Div 2, Group B) dual-head diaphragm type pump. While fresh gas is only introduced into this loop when the

headspace pressure falls below the pressure regulator setpoint (close to atmospheric), a pressure relief valve on the headspace vents excess gas out of the headspace to the outside air in the event that excessive fresh gas is introduced into the reactor. A sample valve is provided in the recirculation loop for periodic sampling of the gas to determine the gas composition. The recirculated gas will likely be saturated with moisture at the bioreactor operating temperature. Since the ambient temperature will be lower than the bioreactor temperature, it is likely that even with insulation and heat tracing, moisture will form in the recirculation line, requiring any instruments to be certified for a saturated gas.

Chemical Feed

Four peristaltic pumps are provided for separate feed of nutrients, NaOH and NH_4OH. The two caustics would not be used at the same time and so it is possible that the same pump could be used for both solutions (swapping out the feed container). Closed-top tanks are used for the nutrient feed and caustic feeds.

Centrifuge

Once a batch run is complete, the bioreactor contents are transferred to a benchtop lab centrifuge. During the run, the centrate will be discharged into the CIP tank for disinfection prior to discharge to the sewer. At the end of the run, the bowl is manually drained prior to another dewatering run.

5.3.2 Compressed Gas Storage, Chemical Storage, and Safety

Hydrogen Gas

H_2 gas is flammable in the range of 4 to 75% by volume and is explosive in the range of 18 to 60% by volume. The International Fire Code (IFC) and International Building Code (IBC) requirements for H_2 storage and handling are a function of the stored quantity in relation to the maximum allowable quantity (MAQ) under the code. The MAQ for areas where hydrogen gas is either stored or used is 1000 standard cubic feet (scf) [Table 6.3.1.1, NFPA 55; Table 2703.1.1 (1), IFC and Table 307.7(1), IBC]. Since the storage quantities in the pilot plant are below the MAQ, most of the code requirements do not apply (except those listed below). The entire process area is considered a "control area" within the pilot building under the IFC 2703.8.3 definition, while the outdoor storage area could be considered a separate control area. Assuming that these are separate control areas, they must be separated by a 1-hour fire rated wall. Furthermore, the floor of the process area must have a 2-hour fire-resistive rating (IFC, 2703.8.3.3). Electrical equipment in and around the area with potentially flammable or explosive concentrations of hydrogen are classified according to the National Electric Code (NFPA 70). The headspace of the bioreactor and the inside of the recirculation piping (and recirculation pump) would be classified as Class I, Div 1, Group B. The process room (which would include the electrical wiring and motors) would be classified as Class I, Div 2, Group B.

Oxygen Gas

O_2 gas is classified as an oxidizing gas, which is a physical hazard. The MAQ for an oxidizing gas is 1500 scf [Table 6.3.1.1, NFPA 55; Table 2703.1.1 (1), IFC and Table 307.7(1), IBC]. The quantity of O_2 gas in storage and use in the pilot plant is also below the MAQ.

General Requirements for Compressed Gases

The following general fire-code requirements must be met, regardless of the quantity of gas:

1. The room must have mechanical exhaust ventilation at a minimum of 1 scfm/sf of floor area, with a shutoff control for the ventilation outside of the use area [NFPA-55, 6.16]

2. The areas where hydrogen and oxygen are stored and/or used must be provided with hazard identification signs [NFPA-55, section 6.12 and IFC, 2703.5]. Since the area will be employed to store and use flammable compressed gases, 'No Smoking' signs must also be installed [NFPA-55, 7.6.4.2 and IFC, 2703.7.1.4]

3. When storage containers have a capacity of more than 5 pounds, incompatible stored materials (such as oxygen and hydrogen) must be separated by a distance of not less than 20 feet, or by a non-combustible partition extending not less than 18 inches above and to the sides of the stored material. [2703.9.8, IFC]. Since the plan is to store a maximum of 5 pounds of each gas, this provision does not apply. If the storage containers were both indoors (and since they are both below the MAQ), the containers would need to be separated by either 50 feet or a 2-hour fire-resistive barrier [NFPA-55, 7.6.3.2.1]. The gas storage and gas mixing will be located outdoors, so this section does not apply.

4. Compressed gas piping must be marked to include the contents name and direction of flow at each valve, floor or wall penetration, and at a minimum spacing of 20 feet [IBC, 3003.2.3]

5. Compressed gas cylinders must be secured against accidental dislodgement and unauthorized access and from falling caused by contact, vibration or a seismic event. Guard posts or other physical means of protection from vehicular damage are required. [IBC, 3003.3].

6. Compressed gas cylinder valves must be protected from physical damage by a protective cap [IBC, 3003.3]

Sodium Hydroxide

A small quantity of NaOH will be used for pH control of the bioreactor during the last part of the batch cycle. NaOH is classified as a corrosive liquid, and the MAQ for this is 500 gallons. Since the quantities stored will be less than the MAQ, most of the IFC requirements do not apply. Those that do apply are:

- Provide hazard identification signs [IFC, 2703.5].

- The separation requirements of the IFC do not apply, since NaOH (a corrosive) is not incompatible with ammonium hydroxide (another corrosive) or hydrogen gas. Under the OSHA regulations (1910.151.c), facilities for quick drenching or flushing of the eyes and body (such as an emergency eyewash and shower) are required where the eyes or body of an employee may be exposed to injurious corrosive materials, such as sodium hydroxide. There is no threshold quantity of corrosive material that triggers this

requirement. Therefore, an emergency eyewash and shower must be located near the chemical use area.

Ammonium Hydroxide

A small quantity of NH_4OH will be used for pH control of the bioreactor during the first part of the batch cycle. NH_4OH would be classified as a corrosive liquid, and the MAQ for this is 500 gallons. Since the quantities stored will be less than the MAQ, most of the IFC requirements do not apply. Those that do apply are:

- Provide hazard identification signs [IFC, 2703.5].
- The separation requirements of the IFC do not apply, since NH_4OH (a corrosive) is not incompatible with NaOH (another corrosive) or hydrogen gas, however as already discussed, an emergency eyewash and shower must be located near the chemical use area.

5.3.3 Container Installation

The current implementation concept for the pilot plant unit is to install the equipment inside a standard 20-foot shipping container, modified to meet the fire code requirements. A layout of the container has been developed (see Appendix B). The container is separated into two rooms — an outer equipment room and an inner bioreactor room — using a stud and gypsum board wall to provide a minimum 1-hour fire rating. A 1-hour fire rated door in the wall provides access into the bioreactor room. The bioreactor room is ventilated with an intake fan (minimum of 35 cfm) and an exhaust louver. The motor of the intake fan would be located outdoors, out of the control area. The bioreactor room is monitored with a H_2 sensor. Additional laboratory equipment for growing inoculum and analyzing the gas would be located at the pilot site. This additional equipment could include a gas chromatograph, a small reactor for growing inoculum, and a small autoclave for sterilization. However, based on the current layout of the pilot, this additional equipment would require a larger shipping container or a second laboratory container.

As discussed above, the floor of the container requires a 2-hour fire rating. Since a typical container floor constructed with a plywood floor material over steel is insufficient, the floor requires modification to be compliant. This could be done, for example, by pouring a thin (4″) concrete slab reinforced with welded wire fabric (WWF) across the floor.

The following external utilities will need to be provided to the container:

- 1-1/2″ water service, primarily to serve the emergency eyewash and shower. Minimum pressure of 50 psig.
- 4″ sewer connection
- Single phase 120 VAC power. A single 30 amp circuit will be sufficient based on current estimate of the cooling loads. An increase in the cooling load would require an upsizing of the circuit rating.

5.3.4 Equipment

A process flow diagram (see Appendix B) and equipment list (not included here) was created for the pilot plant design. The equipment list comprised over 80 pieces of equipment including information on size, rating, manufacture, base cost and additional costs for taxes and shipping. This equipment list included safety equipment (e.g. H_2 gas sensor, emergency eyewash), vessels and tanks (e.g. bioreactor, compressed gas tanks, media tanks, caustics, cleaning solutions), probes and sensors (e.g. pH, ORP, pressure gauges), connections and valves and flow meters, pumps (liquid and gas), motors, heating/cooling units, and centrifuges.

CHAPTER 6:
Pathway Engineering to Create Novel Products

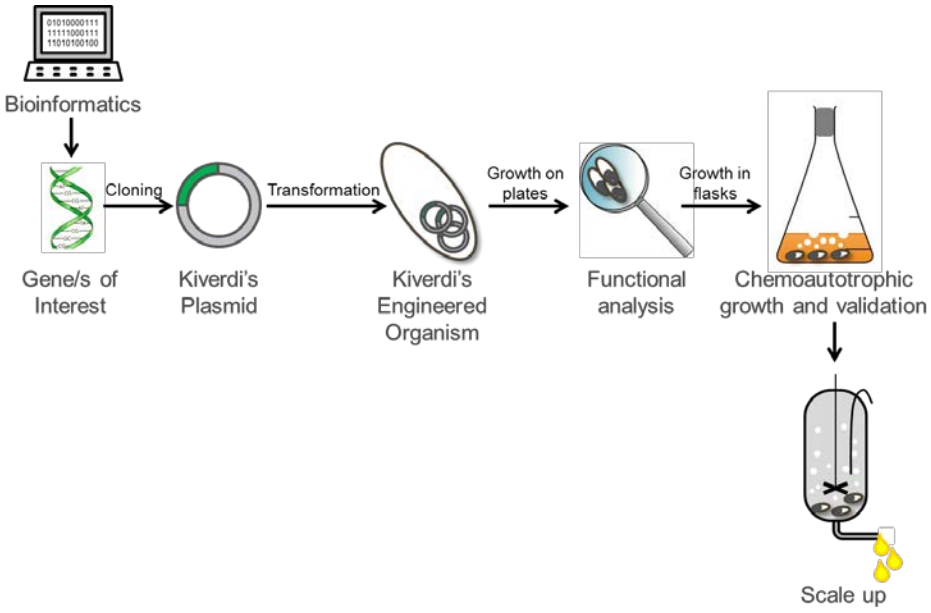
In complementary work outside the scope of this Energy Commission study, Kiverdi has developed methods for engineering *C. necator* in order to generate potentially valuable compounds that are not normally made by the strain. Even though the work occurred outside the project, a description of the results of this work are included in this chapter, as it relates to the overall goal of developing CO_2 conversion to high value chemicals. It also demonstrates how Kiverdi was able to build upon the work that was conducted under the Energy Commission project.

6.1 Engineering Fatty Acid and Hydrocarbon Pathways in Knallgas Microorganisms

The purpose of engineering metabolic pathways in knallgas microorganisms is to transform them into platforms for the production more valuable or desirable chemicals than what they naturally produce, or to increase the yield, titer, and productivity of a given chemical. Although a wide diversity of knallgas strains exist in nature that have evolved many useful physiological and metabolic capabilities, the potential of these microorganisms can be enhanced through modern strain engineering and genetic techniques. For example, the knallgas strain *Cupriavidus necator* grows very rapidly on CO_2 but produces primarily proteinaceous biomass or the natural polyester polymer PHB. Although these materials have potential applications, producing the large amounts of oil chemicals that were sought after in this particular project would likely require modification of the strain.

To increase the range of chemicals that can be produced from CO_2 using knallgas microorganisms, Kiverdi has also been developing a genetic platform to engineer strains based on knallgas microorganisms such as *C. necator* (Figure 33). Metabolic pathway engineering can be used, for example, to change the carbon-number or carbon-chain length of a fatty acid molecule synthesized by the knallgas microorganism. It can be also be used to remove oxygen from fatty acid molecules in order to produce a fatty alcohol or a hydrocarbon. Such approaches can be useful in targeting higher value or more desirable oils. The production of non-native molecules, such as free fatty acids, fatty alcohols, or hydrocarbons, can also increase yield by a phenomenon called metabolic pull. The phenomenon results from the fact that, unlike with natural products, some non-natural products will not be detected by any feedback loops that inhibit production through a given metabolic pathway, such as fatty acid biosynthesis. This can result in a large redirection of carbon through a metabolic pathway, such as fatty acid biosynthesis, without inhibition or feedback, resulting in much higher yields and titers than could be attained for natural molecules produced through the native metabolic pathway.[35]

Figure 33: Platform for Engineering Knallgas Strains



A particular focus of effort has been engineering knallgas strains to increase synthesis of fully deoxygenated, hydrocarbon molecules. Such hydrocarbon molecules are the best suited bioproducts for the production of drop-in fuels such as gasoline, diesel, and jet fuel, since drop-in fuels are generally deoxygenated. In contrast, more traditional but less infrastructure compatible biofuels such as ethanol and biodiesel are oxygenated. This direct biosynthesis of hydrocarbons can be enabled or enhanced through genetic engineering of strains. In producing the hydrocarbons in an entirely biological synthesis reaction, the costs of hydro-finishing in the production of hydrocarbon drop-in fuels can be avoided, and the overall production process simplified. Thus the biological synthesis of hydrocarbon molecules could significantly reduce de-oxygenation costs.

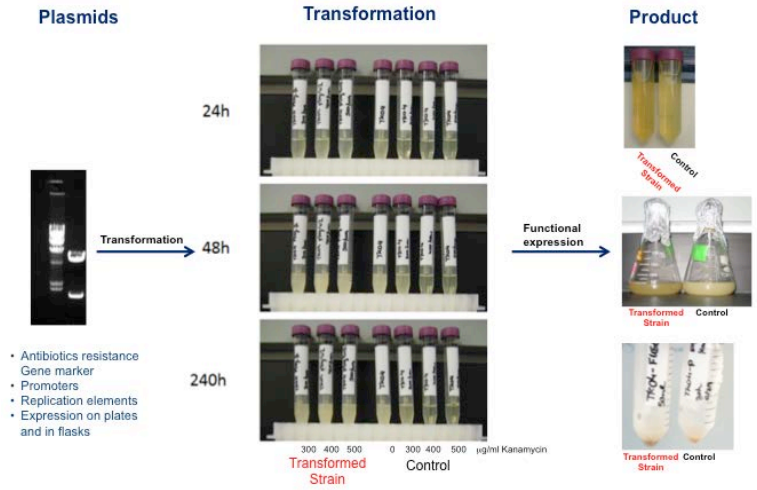
Additionally, deoxygenated hydrocarbon molecules may be more readily secreted from cells and can separate into a non-aqueous phase. A separate hydrocarbon phase can be more cheaply and simply separated from aqueous culture broth than can compounds having greater solubility in water, such as ethanol or butanol, for example. These compounds having greater solubility in water are typically distilled, and may require more elaborate purification techniques such as molecular sieves. Finally, the continuous removal of an insoluble product layer can be used to improve yield, since product inhibition can be lessened or removed. Consequently the biological production of hydrocarbons, if properly implemented, could be more straightforward and less expensive than the production of traditional biofuels such as ethanol.

A performance advantage that biologically produced hydrocarbon molecules could have relative to the traditional sourcing of such molecules from petroleum, is in production of specific, targeted molecular structures. Petroleum is generally a mixture of hundreds to

thousands of different hydrocarbon molecules, most, or all of which, won't have a given structure that is sought after. This is particularly the case when a hydrocarbon molecular structure is sought that does not have a linear carbon-chain backbone. Naturally occurring petroleum hydrocarbon molecules tend to have linear carbon-chain backbones. So too do hydrocarbons produced through F-T, as discussed in the Chapter 1. To increase the quantity of cyclic, branched, or aromatic molecular structures, which are useful for fuels and petrochemicals, isomerization processes are generally required. These isomerization processes are a major activity at oil refineries, and often require complex catalysis, substantial energy expenditure, and lead to loss of product. The direct production of desired molecular structures with relatively high yield and specificity, through a biological synthesis reaction, can reduce costs and pollution associated with isomerization, separations, and purification, compared to a petroleum base case.

To produce increased amounts of hydrocarbons, as well as an array of other potentially useful chemical products, part of Kiverdi's effort has been directed at the development of a biotechnology toolkit for knallgas strains. This has included the development of genetic vectors such as plasmids that can be used to transform knallgas strains with genes of interest. These vectors include antibiotic resistance gene markers for selection of transformants, promotors for gene expression, and replication elements. Kiverdi has also developed protocols for the growth of transformants on plates, and in flasks or gas-tight bottles under selective conditions, such as in the presence of antibiotics. The general scheme from plasmid vector to biosynthesis of targeted product is outlined in Figure 34.

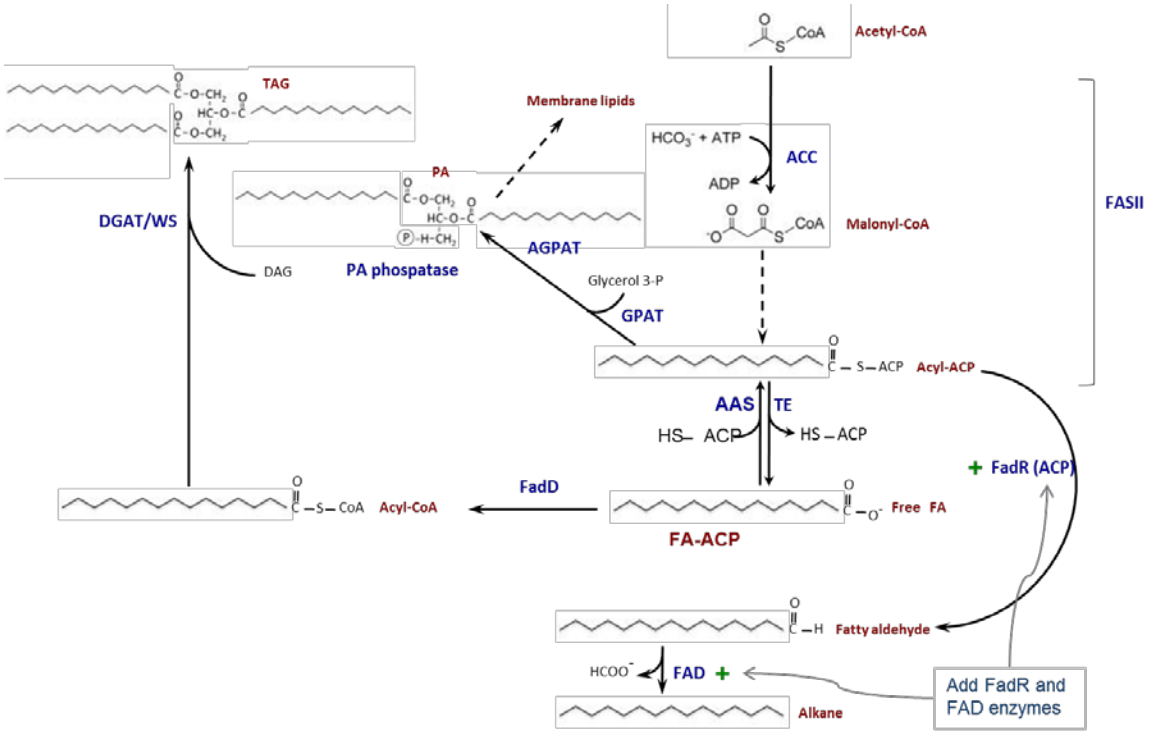
Figure 34: Developing a Strain Engineering Toolkit for Knallgas Microbes



Fatty acid (FA) synthesis from acetyl-CoA precursors is accomplished in bacteria by a type II FA synthase (FASII) - a multienzyme system, utilizing a freely dissociable acyl carrier protein (ACP). The products of FASII are released as acyl-ACPs and may be directly incorporated into membrane lipids by acyltransferases that attach a FA to the glycerol 3-phosphate backbone to form the key intermediate, phosphatidic acid (PA) (Figure 35). In *Cupriavidus necator* acetyl-CoA

is also the central metabolic intermediate that leads to the PHB synthesis pathway, catalyzed by polyhydroxyalkanoate synthetase (PHAS). Consequently, it is apparent that fatty acid biosynthesis competes with PHB synthesis; and in wild type *C. necator*, there is a strong bias towards the production of PHB, reflected by the higher cellular content of PHB than fatty acids.

Figure 35: Biosynthesis of Fatty Acids and Deoxygenation to Hydrocarbons



Source Dr. Christer Jansson LBNL.

Through engineering of the enzymes that control fatty acid chain length (thioesterases or TE in Figure 35), it is possible to alter the chain lengths of the fatty acids in the TAGs produced by knallgas microorganisms. TEs are a type of enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP) during fatty acid synthesis. As an example, TE changes have allowed significant enhancement of the C12 FA, lauric acid, and C14 FA, myristic acid in *C. necator*, where the unmodified organism produced little or none of these chain lengths. As described in the previous chapters, the natural tendency of *C. necator* is to produce primarily C16 fatty acids, such as palmitic acid. Through genetic engineering, Kiverdi has also, conversely, increased fatty acid chain lengths, as well as demonstrated the ability to increase production of totally deoxygenated hydrocarbons in *C. necator*.

A type of modification of the native fatty acid biosynthetic pathways that can lead to a totally deoxygenated hydrocarbon (e.g. an alkane) is illustrated. The metabolic engineering to implement this modification in *C. necator* was performed in collaboration with Dr. Christer Jansson, formerly at Lawrence Berkeley National Laboratory (currently at Pacific Northwest National Laboratory). Independent work on a closely related strain, *Ralstonia eutropha* H16,[36]

validates the general approach. For example, Beller et al. demonstrated the production of hydrocarbons in *R. eutropha* using a genetic engineering strategy similar to that outlined in Figure 6.3,[37] as well as re-engineering fatty acid biosynthesis in *R. eutropha* to produce high value methyl ketones.[38]

6.2 Hydrocarbon Production via Rational Design

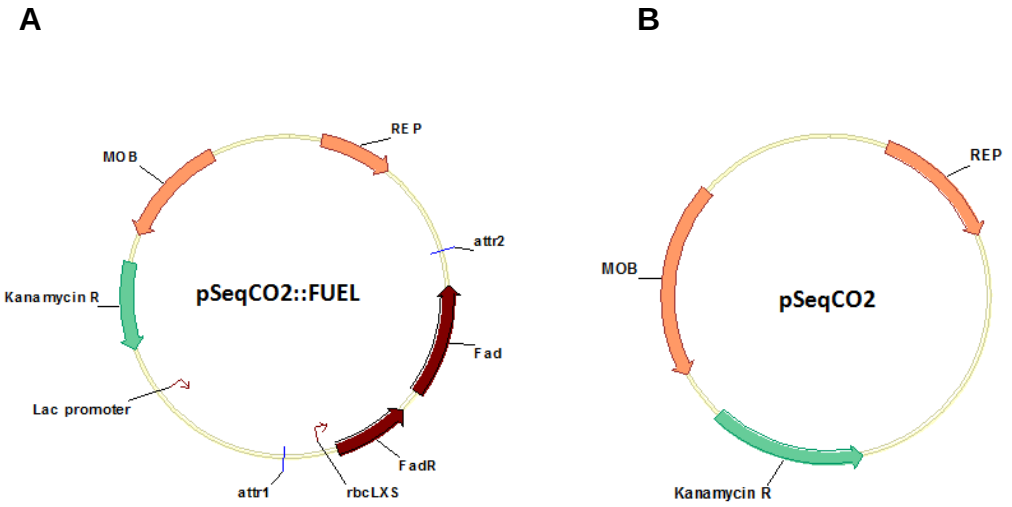
Important insights into the natural synthesis of hydrocarbons by microorganisms have been provided through observations in cyanobacteria. The biosynthesis pathway for hydrocarbon (alkane) synthesis in cyanobacteria appears to proceed via the decarbonylation of fatty aldehydes[39], as illustrated in Figure 35. The decarbonylation pathway implies the involvement of the Fatty acyl-CoA/Fatty acyl-ACP reductase (FadR) and Fatty aldehyde decarbonylase (FAD) enzymes[40, 41]. Gene sequences for FadR and FAD have been identified from several cyanobacteria, and alkane biosynthesis was demonstrated in *E. coli* by utilizing genes for a FadR with high specificity for acyl-ACP (FadR[ACP]) and FAD, both identified by comparing the genomes of alkane-producing and non-alkane-producing cyanobacteria[42].

A strategy similar to what had been demonstrated in *E. coli* was attempted to explore the ability of a knallgas strain to deoxygenate fatty acids to produce hydrocarbons. To redirect carbon flux away from fatty acid and towards hydrocarbon biosynthesis, the genes Fatty acyl-CoA/ Fatty acyl-ACP reductase (FadR) and Fatty aldehyde decarbonylase (FAD) from the decarbonylation pathway of cyanobacteria were expressed in *C. necator*. The result was a major increase in the biosynthesis of hydrocarbons for the transformed strain, as well as a change in the types of hydrocarbon synthesized.[2]

The engineered strain of *C. necator* was made by introducing the *Synechocystis sp*. PCC 6803 FadR and FAD genes driven by the *Synechocystis sp.* Rubisco large subunit mutated promoter. The alkane synthesis genes, provided by Christer Jansson (LBNL), were incorporated into a plasmid vector that can express the genes under antibiotic selection in *C. necator*. The vector with the hydrocarbon synthesis genes and the control vector without these genes are shown in Figure 36. The *Synechocystis sp.* FadR and FAD genes driven by the *Synechocystis sp.* Rubisco large subunit mutated promoter are indicated in brown. The plasmid also contains a kanamycin resistance gene, the IncQ like replication gene (REP) from plasmid pBBR1MCS-2[43] and mobility (MOB) gene for conjugal transfer when RK2 transfer functions are provided *in trans*.

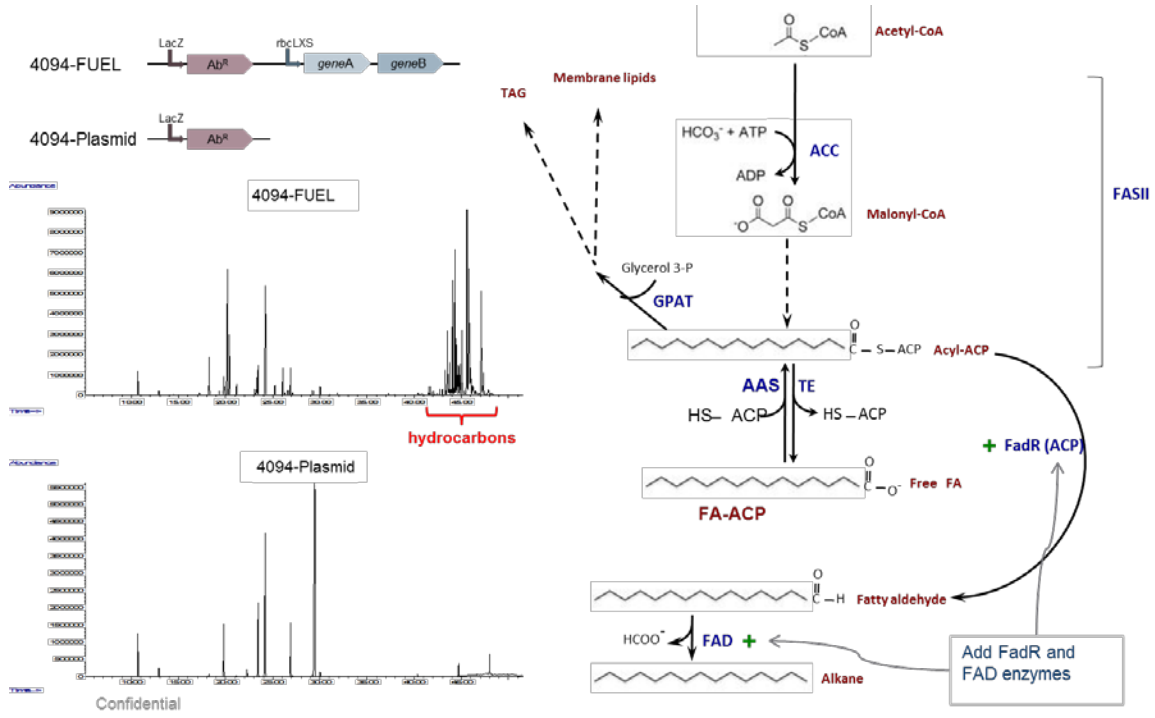
[2] The untransformed strains were found to synthesize small amounts of the hydrocarbon Squalene naturally – a triterpene synthesized through a different metabolic pathway than fatty acid biosynthesis i.e. an isoprenoid pathway.

Figure 36: Plasmid for Expression of FadR/FAD Genes in *C. necator* (A) and Parent Vector (B)



Transformation of *Cupriavidus necator* with the plasmids pSeqCO2 and pSeqCO2::FUEL was carried out by electroporation and transformants were selected after cultivation for 48 h at 30°C on NR-agar plate containing the antibiotic kanamycin (200 µg/ml). Transformants were grown in LB media and lipid samples were extracted from cell pellets for analysis by GC/MS. The chromatograms of the lipid extract from *C. necator* transformed with the FadR and FAD genes (4094-FUEL), and a control (4094-plasmid), which just contained the empty vector, are shown in Figure 37. *C. necator* expressing the *Synechocystis sp.* PCC 6803 genes FadR and FAD showed a prominent set of peaks at 45 minutes that were absent from the control, indicating the production of new compounds. Mass spectrometry analysis of the peaks at 45 minutes indicated the presence of linear and cyclic aliphatic hydrocarbon molecules with carbon chain lengths between 11 to 20 carbons (data not shown). The presence of cyclic hydrocarbons in the products was surprising given the modification of the fatty acid biosynthetic pathway was expected to produce only linear products. The mechanism for this unexpected result is still under investigation. Notably, about 40% of the hydrocarbons produced by the transformation fall within the range of carbon numbers and types of molecular structures appropriate for producing jet fuel.

Figure 37: Production of Hydrocarbons in *C. necator* by Metabolic Engineering



6.3 Fatty Acid Chain-Length Modification via Rational Design

Most bacteria lack intracellular TEs that act on FA-ACPs, and formation of free FAs mainly occurs during recycling of membrane lipids or degradation of acylated proteins. *E. coli* and other bacteria that can take up and metabolize exogenous FAs possess periplasmic TEs (e.g. TesA in E. coli) that liberate FAs for import. Heterologous expression of TEs in bacteria, primarily from plants, has resulted in high production of free FAs[44, 45, 46, 47]. The concomitant decrease in acyl-ACP levels has also been found to relieve the rigorous feedback inhibition of acetyl-CoA carboxylase (ACC; EC 6.4.1.2) (and other FA-biosynthesis enzymes) that is exerted by this end product. ACC catalyzes the rate-limiting step in FA synthesis. Thus expression of TEs in the cytosol of bacteria can have the dual effect of producing free FAs and enhancing FA synthesis by removing inhibition and increasing carbon flux through the FA pathway.

Another area of development in the field has been the targeting of fatty acids having short chain lengths; in the C8 to C14 range. The introduction of TEs favoring the production of shorter chain lengths and the over-expression of TEs to increase shorter chain length fatty acids is one of the means by which the populations of C8-C14 fatty acids have been increased. This approach has been implemented and demonstrated to produce fatty acids having shorter chain lengths in heterotrophic microorganisms that grow on sugar (for example US Patent 7,883,882).

The ability to shift FA chain-length toward shorter chain fatty acids C12 (Lauric acid) and C14 (Myristic acid) was demonstrated *in C. necator* by introducing the TE gene *FatB2* from *Cuphea lanceolata.* The *FatB2* gene was cloned into a plasmid pSeqCO2 under control the Lac promoter

to yield pSeqCO2::TE (Figure 38). Two independent transformants (pSeqCO2::TE1 and pSeqCO2::TE2) were made and lipid samples analyzed by GC/MS. A dramatic increase (>5-fold) in production of fatty acids C12 (Lauric acid) and C14 (Myristic acid) was detected in *C. necator* transformed with the FatB2 TE gene compared to the pSeqCO2 control (Figure 39).

Figure 38: Map of Plasmid pSeqCO2::TE

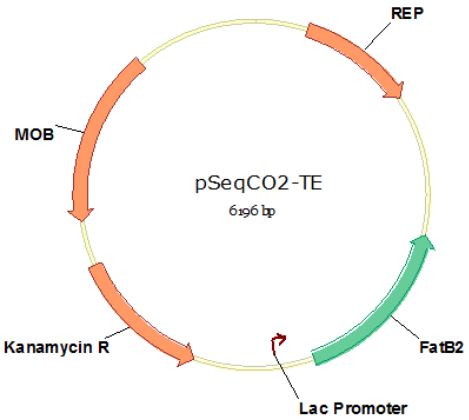
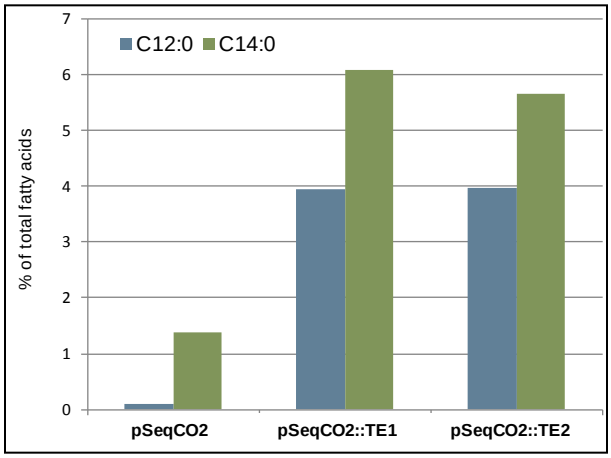


Figure 39: Enhanced Synthesis of C_{12} and C_{14} Fatty Acids in *C. necator*



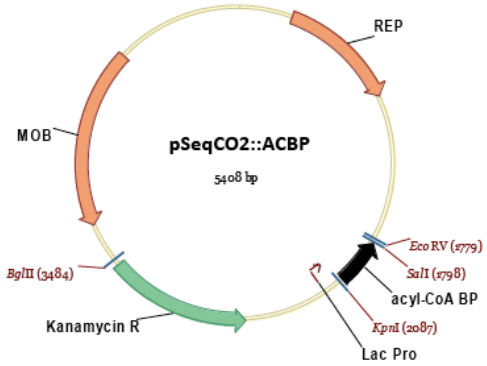
GC analysis of transformants containing the FatB2 TE (pSeqCO2::TE1 and TE2) and control (pSeqCO2).

An alternate and complementary approach was tested to generate short-chain fatty acids that targets the acyl carrier binding protein (ACBP). Like the TE, this protein can act to truncate fatty acid biosynthesis, resulting in the production of shorter acyl chains that can be released as free fatty acids or incorporated into TAGs. ACBPs are known to sequester fatty acyl-CoA esters

from the fatty acid/acyl-CoA synthase machinery and deliver them to enzymes involved in complex lipid biosynthesis. [48, 49]

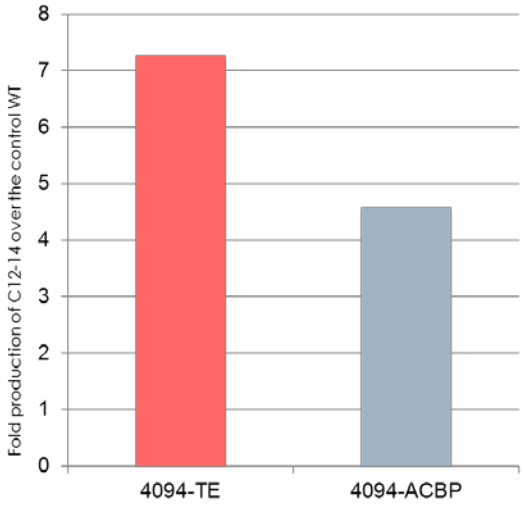
Bos Taurus (cow) ACBP was codon optimized for expression in *C. necator* and synthesized with restriction sites for cloning into pSeqCO2 to give pSeqCO2::ACBP (Figure 40). Transformation of *C. necator* with the plasmids pSeqCO2 and pSeqCO2::ACBP was carried out by electroporation and transformants were selected after cultivation for 48 h at 30°C on NR-agar plate containing the antibiotic kanamycin (200 μg/ml). Transformants were grown in LB media and lipid samples were extracted from cell pellets for analysis by GC/MS.

Figure 40: Map of Plasmid pSeqCO$_2$::ACBP



C. necator transformed with cow ACBP (4094-ACBP) shows a relative increase in C12 and C14 chain length fatty acids comparable to that obtained with overexpression of the FatB2 TE (4094-TE) described above (Figure 41).

Figure 41: Enhanced Production of C_{12} and C_{14} Fatty Acids with Exogenous Fatty Acyl-CoA Binding Protein or TE

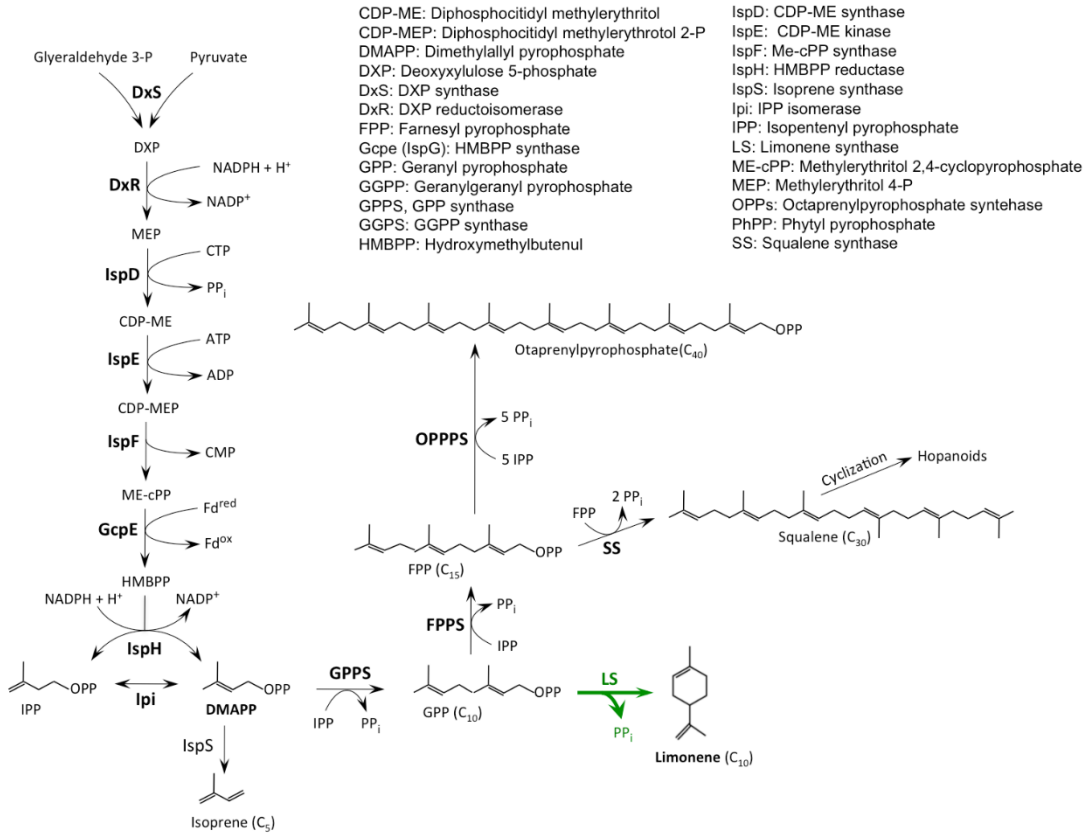


6.4 Engineering Monoterpene Synthesis

Terpenes including monoterpenes are produced through the isoprenoid pathways, which are distinct from fatty acid biosynthesis. Generally speaking, nature uses the fatty acid biosynthesis pathway for the production of mid- to long- linear carbon chains, while the isoprenoid pathways are used for the synthesis of cyclic and branched hydrocarbons and lipids. Monoterpenes are common in plants and algae but are not usually produced in bacteria. Limonene is a $C_{10}H_{16}$ cyclic and branched monoterpene hydrocarbon. The limonene molecular structure is shown in the lower right of Figure 41. It has many current and potential uses, including as a jet fuel blend stock, due to its fuel properties (cloud point, flash point, energy density/content, viscosity etc.), which are compatible with Jet A-1 aviation kerosene. Limonene has also been reported to spontaneously secrete from cells that are engineered to produce it[50]. Limonene is only slightly soluble in water and the molecules in aqueous solution separate into a float layer. Consequently liquid-liquid separation steps for secreted limonene would be minimal.

The isoprenoid pathway that occurs in *C. necator*, starting from the central metabolic intermediates Glyceraldehyde 3-phosphate (G3P) and pyruvate. The native carbon-fixing metabolism (i.e. Calvin cycle) in *C. necator* is naturally able to synthesize the G3P and pyruvate central metabolic intermediates from CO_2. This isoprenoid pathway is known as the non-mevalonate pathway or the methyl-erythritol-4-phosphate (MEP) pathway, and it occurs in plant and algal chloroplasts, and in cyanobacteria and other bacteria including *C. necator*. The branching point in the MEP pathway that leads to limonene through a single enzymatic step is catalyzed by limonene synthase (LS). This enzymatic step, highlighted in green, represents the only enzymatic step that needs to be added to *C. necator*'s native machinery for the limonene molecule to be synthesized from the central metabolites G3P and pyruvate through the MEP isoprenoid pathway. Bioinformatics analysis supported the existence of the MEP pathway within *C. necator*. Furthermore, GC/MS analysis of *C. necator* extracts indicated the presence of the triterpene squalene (molecular structure middle right), confirming the active expression of the MEP pathway in *C. necator*, and the ability of this strain to biosynthesize terpene molecules through the MEP pathway.

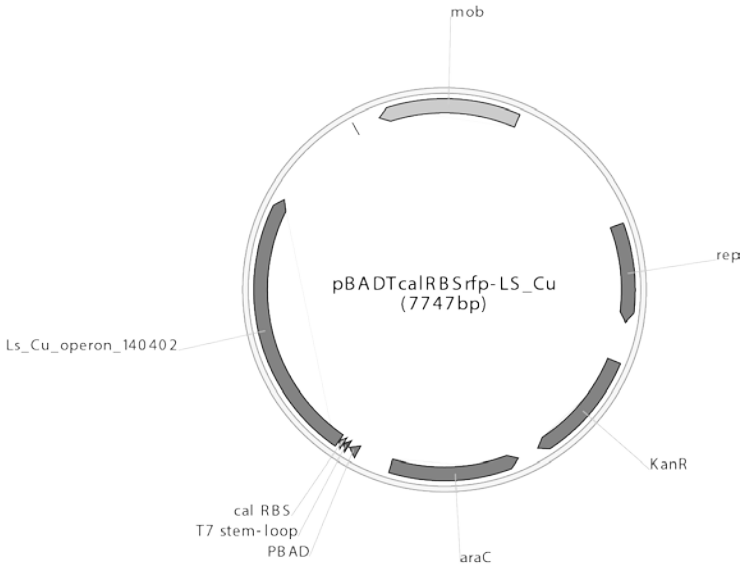
Figure 41: The Methyl-Erythritol-4-Phosphate (MEP) Pathway with Conversion to Limonene



Source: Jansson, Progress in Botany 73:81 (2012).

Experiments were performed to express a limonene synthase (LS) gene in *C. necator* using the plasmid pBADTcalRBS and under the control of the ara promoter. Limonene synthase from *Citrus unshiu* (Uniprot Q6F5H3) was codon optimized (LS_Cu) for expression in *C. necator* and cloned into the vector pBADTcalRBS[51]. Plasmid pBADTcalRBS-LS_Cu is depicted in Figure 42. Transformation of *C. necator* with pBADTcalRBS-LS_Cu was carried out by electroporation and transformants were selected after cultivation for 48 h at 30°C on NR-agar plate containing the antibiotic kanamycin (200 µg/ml). Transformation was confirmed by isolating plasmid DNA from a 3-mL overnight LB culture containing 200 µg/mL kanamycin and sequencing the isolated plasmid to confirm the presence of the gene.

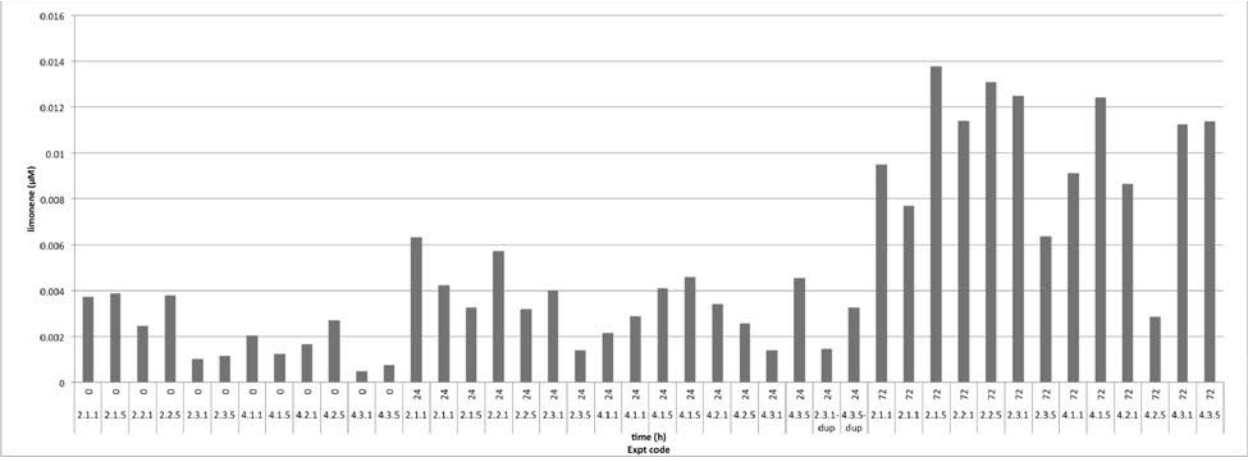
Figure 42: Plasmid Used to Express *Citrus unshiu* Limonene Synthase in *C. necator*



Cultures of two *C. necator* transformants with pBADTcalRBS-LS_Cu were grown as follows: A small amount of cells from the patch plate were used to inoculate 3 mL of LB media containing 200 μg/mL kanamycin. The culture was incubated overnight at 30 °C. A 50-μL aliquot of the overnight culture was used to inoculate 5 mL of LB containing 200 μg/mL kanamycin in a glass culture tube. The media was overlaid with 500 μL of dodecane and incubated at 30 °C with shaking (250 rpm). After six hours of incubation, arabinose was added to 0.1% (w/v) or 0.5 % (w/v). A 100-μL aliquot of dodecane was removed at 0, 24, 72, and 144 hours post-induction. After the 144 h timepoint was collected, 200 μL of fresh dodecane were added to the culture. At 192 hours post-induction, a final aliquot of 100 μL was collected.

Samples were prepared for analysis by diluting 50 μL of the dodecane layer removed from cultures in 300 μL of ethyl acetate. Limonene was detected on an Agilent 6890N GC/MS with a 5975C MS detector (Santa Clara, CA). The column used was a Cyclosil B (J&W Scientific, 30 m x 320 μm), injection temperature 250 °C operating in splitless mode. The column flow rate was 1 ml/min, with an initial temperature 60 °C, a ramp 10 °C/min to 135, and a ramp 30 °C/min to 200. Data acquisition was in SIM mode, ions monitored were 68 and 93. Quantification was accomplished by running known standards of D-Limonene (Sigma). Limonene produced by the cultures is shown in Figure 43.

Figure 43: Limonene Production in *C. necator* Transformed with *Citrus unshiu* Limonene Synthase



The x-axis is labeled with time (h), (transformant), (replicate), (arabinose concentration).

6.5 Conclusions and Next Steps

Genetic tools to engineer the knallgas strain *C. necator* were developed by introducing genes into *C. necator* which shift the carbon-chain length distribution of fatty acids; and that produce hydrocarbons through modifications to the fatty acid biosynthesis pathway, as well as through modifications to an isoprenoid pathway. As described, it has been successfully demonstrated in transformed *C. necator* the ability to:

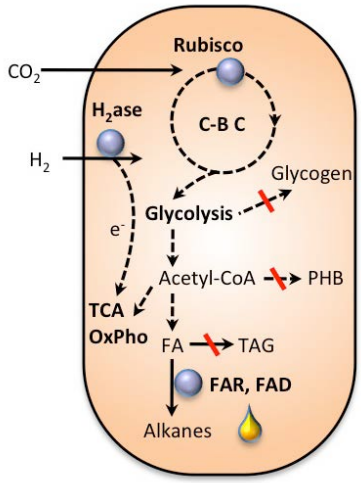
1. Grow transformants under the selection pressure of the antibiotics.
2. Produce shorter-chain fatty acids using the thioesterase gene (*FatB2*) as well as using the ACBP gene.
3. Produce hydrocarbons through the fatty acid biosynthesis pathway using the FUEL operon (*FadR and FAD* genes), and through the MEP pathway; including native production of the triterpene hydrocarbon squalene, and engineered production of the monoterpene limonene.

The functional expression of the genes and production of hydrocarbons (and absence of production in the wild type and empty plasmid controls) was confirmed at LBNL using GC/MS. The increase in short-chain fatty acids compared to the wild type was confirmed at LBNL using GC/MS and by the outside contract laboratory Eurofins Scientific (Metairie, LA) using GC protocols. The technology and tools developed in this work for *C. necator* can enable the targeting of genes for functional expression to convert CO_2 into value added products including fuels and petrochemical replacements.

The initial demonstration of various genetic modifications that have been described here can be further improved upon by a combination of approaches including codon optimization, selection of suitable strong promoters, and overexpression of targeted genes/enzymes in the relevant pathways. Competing pathways that direct carbon away from the desired targeted product (e.g.

PHB synthesis) can be suppressed through methods such as chromosomal deletion of crucial genes along competing pathways (Figure 44). Next steps should include the design and characterization of several promoter systems for *C. necator* that allow for varying levels of inducible gene expression, and the ability to independently manipulate the expression of multiple enzymes. This type of capability is important for the optimization of multi-gene synthetic pathways. The production of hydrocarbons or short-chain fatty acids could be further enhanced in transformed *C. necator* by expression of the optimized hydrocarbon biosynthesis genes and thioesterase, and the suppression of genes involved in carbon flux toward accumulation of poly-D-3-hydroxybutyrate (PHB) such as the PHAS/operon for PHB synthesis Figure 6.13. An increase in the production of hydrocarbons having middle distillate carbon chain lengths, and properties particularly suitable for use in jet fuels, could be achieved by co-expression and/or overexpression of the FUEL operon along with thioesterases having high affinity for C8-C12 FAs and/or the ACBP genes.

Figure 44: Strategies for Enhancing Liquid Hydrocarbon Production from Gas in *C. necator*



Source: Dr. Christer Jansson LBNL.

With respect to the production of monoterpenes, and limonene in particular, next steps could follow the approaches that have been used to increase production of limonene in the biotechnological model strain *E. coli*. The near term objective in adopting and applying these methods to a knallgas strain would be: a) to develop a CO_2 or syngas-utilizing, terpene-producing, biocatalyst; and b) having production optimized at a base-case industrially-relevant bench- scale bioprocess. Early work in *E. coli* gave monoterpene (limonene) titers of 5 mg/L using the endogenous non-mevalonate (DXP) pathway.[52] This early work was improved upon by the installation in *E. coli* of the *S. cerevisiae* mevalonate (MEV) pathway for isoprenoid production. Installation of this pathway into *E. coli* resulted in orders-of-magnitude improvements in isoprenoid yields. Up to 0.4 and 1.0 g/L of limonene and α -pinene, respectively, have been reported.[53, 54] and 27.4 g/L of the sesquiterpene amorphadiene have been reported.[55] Development of a genetic toolbox for *C. necator* would allow for similar

manipulations of IPP/DMAPP flux by introducing, for example, the MEV pathway and/or increasing the constitutive expression of the native *C. necator* MEP pathway. While precedence does not guarantee success, these prior results with *E. coli* show that elevated flux through an over- expressed, recombinant isoprenoid pathway is possible.

It is believed that knallgas microorganisms such as *C. necator* represent an ideal microbial host for the implementation and application of many different metabolic capabilities that have been established in more traditional model platform strains of biotechnology such as *E. coli* and yeast. These metabolic capabilities include the engineered production of hydrocarbons and oleochemicals through modified fatty acid biosynthetic, as well as through isoprenoid pathways. Applying knallgas strains towards this end offers a number of practical advantages for energy and industrial bio-applications that differentiate knallgas strains from common platform microbes like *E. coli* or yeast. Platform microbes are largely favored for their mature molecular biology, genetics, and ease of culturing. While these advantages are valuable, the idea of one-microbe-fits-all has proven limiting in many cases. The advantage of using knallgas strains, versus more traditional model platform strains, lies in the great ability to tap into enormous renewable and sustainable feedstock opportunities, including CO_2, enabled by the highly evolved and optimized chemoautotrophic metabolism of knallgas microorganisms, as discussed in the Introduction chapter. In particular, it enables the use of CO_2 as a feedstock for the production of oleochemicals and middle distillate hydrocarbons.

A compelling case can be made for developing knallgas microorganisms such as *C. necator* as a new type of model platform microbe in biotechnology because of favorable reaction thermodynamics and kinetics, biological capabilities, the required scales, and bottom-line economics. However, one of the more technically challenging aspects of establishing a knallgas strain as a model organism is the relatively limited set of tools currently available for genetic manipulation of knallgas species. Consequently, new genetic tools will need to be developed and the overall toolbox expanded. One can screen genes of interest for the production of targeted chemicals and fuels from CO_2 and identify high-priority candidate genes for metabolic engineering efforts. The team developed a broad, well-characterized synthetic biology toolbox for strains such as *C. necator*, which includes plasmid-based gene expression as well as modifications to chromosomal DNA. These tools will allow for the broad metabolic engineering of a knallgas host organism such as *C. necator* using state-of-the-art systems biology methods to produce an array of targeted bioproducts from CO_2, including high energy density biofuels.

The field of genetic engineering has made remarkable strides in expanding the portfolio of organisms that have been genetically characterized, and which can be modified and improved. The field continues to generate advanced tools for enabling successful bio-engineering of even difficult to grow extremophiles. These technologies can be leveraged in the future for metabolic engineering of knallgas strains, and hydrogenotrophs more broadly, for the production of a range of chemicals and fuels from CO_2 and renewable energy sources. The development of genetic tools and a new type of knallgas platform microbe, will be accompanied by demonstration of this technologies applicability and utility by producing desired fuel and chemical molecules from CO_2 or syngas.

Kiverdi will continue to build its in-house capability in the area of knallgas microorganism engineering as well as pursue partnerships in the biotechnology field with leading laboratories such as LBNL. Kiverdi will also partner in the private sector with companies specializing in biotechnological tool development. Building upon in-house capabilities will include acquiring further equipment, experience, and expertise for growing, scaling-up, and genetically manipulating knallgas microbes. At least initially, the new genetic and molecular techniques developed for knallgas strains are expected to be less efficient than more mature technologies used in model *E. coli* or yeast strains. In the long term, the goal would be to achieve a similar level of efficiency and versatility for genetic modification in a knallgas strain, such as *C. necator*, as now exists for *E. coli* or yeast; and to add a knallgas strain, as well as other types of hydrogenotrophs and carboxydotrophs, to the portfolio of model organisms in biotechnology.

The successful development of genetic tools in a technological platform for a knallgas microorganism would simultaneously address several obstacles to the cost-competitiveness of current biofuels and biochemicals technologies. The ability to use gasified feedstocks, enabled by knallgas microorganisms, means that low cost or waste substrates, which are often heterogeneous, and recalcitrant, can be converted through established gasification technologies into a homogenous gaseous input for the production of high value oleochemicals and fuels. This approach can greatly increase the feedstock options for renewable chemical and biofuel production. The feedstock options increase still further when considering the capture of waste CO_2 from point emission sources using renewably generated H_2.

Other obstacles addressed through the use gas-growing knallgas microorganisms in place of sugar-utilizing model strains such as *E. coli* or yeast including decreasing the likelihood and risk of bioprocess contamination by environmental microbes. It also can decrease the likelihood and risk of escape of a genetically modified microorganism into the surrounding environment, particularly if an obligate chemoautotroph is used, because the H_2 electron donor required for survival and growth is essentially absent from Earth's ambient surface environment. In contrast sugar-growing or photoautotrophic organisms such as algae and higher plants have readily available growth substrate on which to survive and propagate in the surrounding ambient environment. Finally, this biological approach to the conversion of gaseous feedstocks to liquid fuels or chemicals can bypass the sensitivity to gas impurities, and CAPEX challenges, of traditional thermochemical catalytic methods for such gas-to-liquids conversions, such as F-T. The chemoautotrophic conversion of CO_2 or syngas to mid- and long- carbon-chain molecules has great promise as a platform method for the scalable, low-cost, renewable production of biofuels and oleochemicals; and is not currently represented in the commercial biotechnology space.

CHAPTER 7:
Conclusions and Future Development

7.1 Summary of Project Work and Results

7.1.1 Project Motivation and Goals

The overall goal of this project was to develop technology for CO_2 capture and conversion to biological oils, oleochemicals, and fuels using knallgas bacteria; chemoautotrophic microorganisms that can fix C1 molecules such as CO_2 or CO into oil molecules using H_2. The technology is intended to enable conversion of CO_2, and production of biological oils, oleochemicals, and biofuels, with minimal competition or impact on land usage, water usage, food production, or natural habitat. The CO_2 or CO utilized in the process can be sourced from industrial flue gases, exhaust, or syngas. Leveraging established steam reforming or gasification technologies to generate H_2 and/or syngas, the technology can be used to convert low cost, abundant carbon-based resources such as agricultural residues, MSW, wood waste, industrial waste gases, or biogas, into valuable chemicals or fuels. Leveraging established electrolysis technologies to generate H_2, the technology can be used to capture CO_2 from industrial point emission sources and convert the CO_2 into valuable chemicals or fuels. This process design concept provides a promising potential to convert CO_2, biomass and many other carbon-based feedstocks with high carbon efficiency as well as lower cost. Oils produced through this process can be used in the manufacture of drop-in replacements for current petrochemicals and oleochemicals at a lower cost and with better pricing and supply stability than incumbent sources.

The knallgas strains being researched for this application represent a relatively unexplored and emerging area that warrants further investigation and optimization to realize their potential. The knallgas strains are being used to implement bioprocesses based on anabolic biosynthesis pathways such as for fatty acids, amino acids, or terpenes, which can enable the production of higher carbon number products than incumbent chemoautotrophic bioprocesses using H_2 or CO. The use of knallgas strains enables the use of O_2 as a strong electron acceptor in respiration, which increases the efficiency of anabolic biosynthesis. Since knallgas microorganisms do not rely on light to perform the CO_2-fixation reactions, the process can be readily scaled using conventional industrial bioprocessing technology. This is in contrast to photoautotrophic systems using algae for CO_2-fixation. Algal approaches require unconventional photobioreactors constructed out of thin, transparent, materials, which have proven to be uneconomic; or growth in wide open, unprotected ponds. The proposed knallgas technology is predicted to be cost-effective and energy-efficient. These rationales form the foundation and innovation of the research performed through this project.

The experiments presented in this report demonstrated that a capture and conversion system using knallgas microorganisms can produce oils from CO_2 as sole carbon source, including oils that can be converted into biodiesel fuel. The project further aimed to demonstrate that production of chemicals and fuels from CO_2 using knallgas microorganisms can be economically feasible and scalable. Although enhancements in performance are likely required

to achieve desired commercial metrics, the work described here indicates that such goals are within reach and the path to further developing the technology to reach these metrics is discussed below.

7.1.2 Review of Project Work and Accomplishments

This work demonstrated proof-of-concept for CO_2 to oil conversion using knallgas microorganisms combined with subsequent product separations and purification steps. Kiverdi demonstrated its process at lab scale, including culturing lipid accumulating knallgas microorganisms on H_2 and CO_2 gases in a bioreactor, followed by downstream processes - cell harvesting, lipid extraction, and oil purification steps - similar to those commonly used for the recovery of biological oils from algae or yeast (Figure 45). Kiverdi developed methods and protocols for growing knallgas microbes on gaseous energy and carbon substrates, H_2 and CO_2. Protocols for system inoculation, sampling, harvesting and analysis of biomass product were developed for both small scale high-throughput work for screening various strain candidates, as well as at larger scales to produce oil samples from CO_2. The gas bioprocesses were scaled from serum bottles to 20 L bioreactors. Work in many different areas of expertise was performed during this project including in microbiology, strain screening and characterization, bioreactor design, and process design and scale-up. In the course of this work, Kiverdi and its partners acquired considerable experience growing knallgas strains on gas substrates. Many different knallgas strains were characterized and analyzed leading to the development of a strain database with information on knallgas strains including microscopic cell morphology, colony morphology, biomass analysis, oil analysis, and growth characterization.

Figure 45: Knallgas Strain *C. necator* Growing on H_2 and CO_2 Gas and Extracted Oil

For the nearly all aspects of Kiverdi's CO_2-to-oil conversion system - gas pumping and delivery; bioreactor microbial culturing; mass-liquid separation for cell mass removal from the process stream; biomass drying; lipid separation; transesterification of oils, and analysis of oil product by GC/MS - standard proven equipment, consumables, and materials from the chemical and biotechnology industries were used. For the CO_2-free or CO_2-neutral generation of H_2 used in the process there are commercial and experimental technologies available. For testing, the team

initially used purchased H_2 gas supplies, as well as H_2 generated from water using commercially available electrolysis equipment.

Details of the system design and experiments have been described in this report, including the gas delivery system, the knallgas strains and culture environment, the monitoring and control instrumentation, the harvesting system, and the biomass fractionation and analysis. The knallgas microorganisms were grown in standard stirred-tank bioreactors on H_2 and CO_2, supplemented with O_2 (air) and inorganic mineral nutrients (e.g. nitrogen, phosphate, and potassium). These mineral nutrients are similar to those comprising fertilizers typically used to grow plants. H_2 and CO_2 served as the sole source of carbon and energy, respectively, for the growth and biosynthesis of products by the knallgas microorganisms.

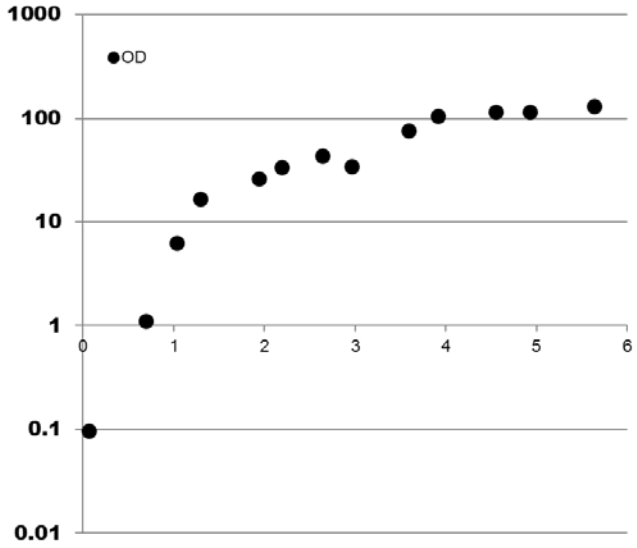
Parameters relevant to the growth and performance of the knallgas microorganisms were monitored and recorded during the experiments, including the cell dry weight density (the amount of dry cell mass per volume of liquid), growth rates (the increase in cell dry weight density per unit time), lipid content and profile; chemical product concentrations, temperature, pH and pH adjustments, mineral nutrient concentrations and additions, dissolved oxygen (DO) levels, redox potential, ammonium levels, anionic concentrations (e.g. phosphate), gas addition rates, inlet gas composition (H_2, CO_2, and O_2 content), headspace gas composition, gas pressure, gas and liquid retention times, agitation rate, and the emergence of any contamination during the course of a run. The information collected on experimental runs was used to improve and optimize the performance of subsequent runs.

Best practices for using toxic and flammable gases in the growth of knallgas microorganisms were implemented, developed, and advanced in the course of this project through following the integrated safety management system developed by the United States Department Of Energy (DOE).

Process optimization and scale-up involved the transfer and adaptation of methods for growing knallgas strains on H_2 and CO_2 from 160 mL gas-tight serum bottles up to 20 L bioreactors. Batch and continuous bioreactor runs were performed.

A procedure was developed to grow the knallgas strain *Cupriavidus necator* on a H_2/CO_2 gas mixture to densities as high as 41 grams dry cell weight per liter (Table 4.3) and OD over 100 (Figure 46), which are within range of typical industrial microbial bioprocesses. Over the course of batch runs that typically lasted on the order of six days, average biomass productivities up to 6.9 g/L/day were attained with instantaneous productivities of up to 17 g/L/day observed during the fastest growth intervals. Biomass doubling times as low as 3-4 hours were observed during the exponential phase of growth, which is faster than autotrophic growth on CO_2 observed for photosynthetic microorganisms.

Figure 46: *C. necator* Grown to Over OD 100 on H_2 and CO_2



To provide a comparison with more well-known organisms that can grow on CO_2 – microalgae – the average areal biomass productivity in the U.S. for algae grown on CO_2 is reported to be 13.2 g/m^2/day.[56] Assuming a typical pond depth of ~0.1 meters (~10 centimeters), this corresponds to a volumetric productivity of around 132 g/m^3/day or 0.132 g/liter/day. The 6.9 g/liter/day average biomass productivity observed for *C. necator* growing autotrophically on CO_2 was therefore around fifty times higher than the autotrophic volumetric productivities typically reported for algae grown on CO_2.

As will be discussed subsequently, there are relatively straightforward bioreactor design changes, and process intensifications, that it is believed will further increase the volumetric productivity of knallgas systems; and the advantage in productivity on CO_2 over photoautotrophic algal systems. As will also be discussed subsequently, it is expected that simply scaling up the reactor sizes, and specifically scaling-up vertically, will enhance mass transfer of low solubility gases such as H_2 and O_2, through increases in hydrostatic pressure as the depth of the water column increases. It is quite plausible that knallgas process performance will only improve with scaling-up vertically.

There is an enormous potential advantage in terms of land usage and land footprint for chemoautotrophic knallgas systems compared to photoautotrophic systems. This advantage becomes apparent when one considers the potential production per unit area of comparable systems. The elevated volumetric productivity of chemoautotrophic systems versus photoautotrophic systems, combined with the far greater facility in vertical scaling of chemoautotrophic systems, can result in massively higher productivity per unit reactor area and unit land footprint.

To get some idea of potential productivity per unit land footprint of a knallgas system at commercial scale, it should be noted that commercial bioreactors, which can be readily applied to the cultivation of knallgas microorganisms, are commonly on the order of ten meters deep, and bioreactors with water columns of up to 40 meters in depth have been applied in commercial operations.[57,58] As already discussed, because of light blockage, algal ponds are typically limited to about 10 centimeters in depth. If the average autotrophic productivity of 6.9 g/liter/day achieved in this project were scaled up to reactors with water columns 10 to 40 meters in depth, this would correspond to areal productivities of 69,000 to 276,000 g/m^2/day, (for the 10 and 40 meter depth cases, respectively). This represents a 5,000 to 20,000-fold advantage in areal productivity over that of algae due to the combination of higher volumetric productivities and vertical scaling. Since biomass productivity is proportional to CO_2 uptake in autotrophs, such as both knallgas microorganisms and algae, this would also be equivalent to around a 5,000 to 20,000-fold advantage in CO_2 capture per areal footprint. In the experimental chapter, an empirical ratio of biomass produced per CO_2 consumed for *C. necator* was determined to be 0.61-0.65 grams of biomass synthesized per gram CO_2 consumed. Assuming this ratio held at the large scales being considered, areal biomass productivities of 69,000 to 276,000 g/m^2/day would correspond to areal CO_2 capture rates of roughly 110,000 to 440,000 g CO_2/ m^2/day.

Improvement in volumetric productivities through straightforward bioreactor design changes and process intensifications, combined with enhanced mass transfer of low solubility gases such as H_2 and O_2, through the increase in hydrostatic pressure that accompanies vertical scaling, could even further increase the areal productivity and CO_2 capture advantage of knallgas systems over phototrophic algae. It not unreasonable to expect the potential advantage in areal productivity and CO_2 capture rate to increase by yet another order of magnitude compared to algal systems through a combination of design improvements and the beneficial impact of increased hydrostatic pressure with scale up.

Algae itself can have a two to 20-fold areal productivity advantage against higher-plant agricultural crops such as soy or oil palms.[59] So a knallgas system could potentially have at least a 10,000-fold advantage in areal biomass productivity and CO_2 capture over a traditional agricultural system. This illustrates the disruptive potential of knallgas systems for the intensification and compactification of biological CO_2 capture and biomass production. Through this process intensification and compactification, the land usage and footprint, and environmental disruption associated with traditional agriculture, can be dramatically lessened, while still producing the same types and quantities of biologically derived products from CO_2.

Although oil production was the focus in this work, other interesting biochemicals were produced from CO_2 that may serve as useful co-products, if produced in an oil production process; or which could represent opportunities on their own. As mentioned, one product that was generally produced in large amounts by the knallgas strain *C. necator* was PHB and other related polyhydroxyalkanoates (PHAs). This natural polyester polymer was commonly found to comprise up to 60% by weight of the cell mass produced from CO_2. In addition, the knallgas strains produced all of the essential amino acids. A particular mutant of *C. necator* was identified that produced higher amounts of lysine, which is an important amino acid in animal

feed. During some periods in the course of bioreactor runs in which *C. necator* cultures experienced anaerobic conditions, some interesting fermentation products resulted. These fermentation products included 1,3-butanediol. 1,3-butanediol is a useful feedstock for the production of synthetic rubbers such a polybutadiene and acrylonitrile butadiene styrene (ABS).

Using the results of small bottle and lab scale bioreactor experiments, a design was created for a mobile pilot scale process to test CO_2 bioconversion using Kiverdi knallgas microorganisms at various locations. The design includes a 50 L skid mounted bioreactor and processing equipment housed within a standard shipping container for transport.

In related work outside the scope of this project, but supported and enabled by the work of this project, Kiverdi developed strain engineering tools for *C. necator*. These strain engineering tools were used to demonstrate the production of high energy density and high value hydrocarbons by *C. necator*. Since *C. necator* naturally exhibits robust growth on H_2 and CO_2, it is thought to represent a useful starting point for engineering new, enhanced CO_2-utilizing strains. Currently, there is a lack genetic tools, generally, for chemoautotrophic organisms that can perform conversions of CO_2 or syngas. The methods and tools developed for *C. necator* will likely be transferable to other knallgas strains identified through the screening activities of this project. This will increase the number of industrial biocatalysts available to produce natural and non-natural chemicals from CO_2 and/or syngas.

Through the strain and process development work of this project, Kiverdi has initiated development of a platform for producing from CO_2 a diverse array of targeted oil molecules having a wide range of carbon chain length, functional groups and bond saturation. Such a platform technology comprising strains, genetic tools, and bioprocess design, for the chemoautotrophic conversion of $H_2/CO/CO_2$ gas mixtures, through various anabolic biosynthetic pathways, to a wide array of potential organic chemical and fuel products, is not currently represented in the biotechnology industries portfolio.

7.2 Recommended Improvements and Next Steps

7.2.1 Additional Design Aspects for Investigation

The experimental systems developed and used in this project were sufficient for characterization of a variety of different knallgas strains when grown on H_2 and CO_2, as well as the scaling up of the bioprocess within the cost constraints of the project. However, due to time and resource limitations several design elements envisioned for a commercial process were not evaluated. One such major design element necessary for commercial process is the recirculation of unused gases which follows from the low solubility and relatively low conversions of the H_2 and O_2 gases with passage through the bioreactor working volume. Additionally the experimental systems used were not optimized for mass transfer of low solubility gases like H_2.

Other aspects that were not addressed in the course of this work include optimizing energy efficiency, maximizing gas conversion, and minimizing capital and operating costs. Such optimization work would be part of a pre-commercial pilot scale development and would likely require customization of bioreactor equipment and designs specifically tailored for the growth of knallgas strains. As mentioned, in this project, off-the-shelf bioreactor equipment was

utilized, which is generally designed for lab-scale sugar-based heterotrophic processes close to ambient pressures and temperatures, such as growing *E. coli* or yeast. Improved performance could be expected using bioreactor designs customized to the unique requirements of knallgas strains and bioprocesses. Elements of the process that could be improved with readily straightforward design modifications include tailoring the aspect ratio (height:width) of the bioreactor as compared to the standard off-the-shelf equipment, reducing the bubble size of the input gas, and increasing the operating pressures; all of which could improve gas dissolution and mass transfer into solution.

7.2.2 Ongoing Areas of Improvement

Enhancing Mass Transfer of Gases

In this project mass transfer of low solubility of H_2 and O_2 gases generally limited the knallgas bioprocess performance. Gas bubbles sparged into the base of the bioreactor rise rapidly through the liquid broth and coalesce in headspace. This rapid partitioning of gas phase from liquid phase reduces gas-liquid interface and mass transfer and increases the gas recirculation required for acceptable conversion of feedstocks. Therefore, increasing gas retention time within the liquid volume should increase H_2 and O_2 conversion. Increasing the gas pressure will improve mass transfer into solution as well increase the thermodynamic driving force of the knallgas metabolism towards products.

Kiverdi plans to continue with implementation of design elements not sufficiently addressed in the current studies including: gas recirculation, particularly for the unconsumed H_2; improving gas transfer with a taller reactor column; adding additional positive pressure to the system, above the hydrostatic pressure; generating smaller bubbles in gas sparging (greater surface-to-volume ratio), thus increasing K_La and flux of gases into solution; and testing alternative reactor designs for improved K_La at a given power input.

The effect of temperature on gas transfer into solution also merits investigation as it would appear to involve conflicting tendencies. Specifically when considering the mass transport equation

$$\frac{dC}{dt} = (K_L a)_f (C^*_{\infty,f} - C_L)$$

The equilibrium concentration term (C*) of gases like O2 and H2 will drop as the temperature increases. This acts to decrease the flux of gas into solution. However the diffusion transport coefficient in K_L will tend to increase with increasing temperature. This acts to increase the flux of gas into solution. It isn't immediately clear which of these counteracting factors would dominate, however a number thermophilic knallgas microorganisms are known to have very short biomass doubling times, as low as one hour, including one of the knallgas strains studied in this project. These are among the shortest doubling times observed for an autotroph growing on CO_2 as sole carbon source. The effect of temperature on the mass transport of gaseous nutrients into solution, and whether higher productivities can be achieved by applying thermophilic strains in place of the mesophilic strains that were the primary focus of this project, merits further investigation.

There are many alternative reactor designs used in the chemical industry that are more effective with gas-liquid reactions than the stirred tank reactor (STR) type design used in this project. Kiverdi is in the process of investigating such alternative gas-liquid reactors used in the chemical industry that have generally not seen application in bioprocessing. The strong dependence of the knallgas bioprocess on gas-liquid interactions suggests alternative reactor designs optimized for gas-liquid reactions could lead to better performance. Kiverdi has begun constructing and testing alternative bioreactor designs against standard STR bioreactor designs to evaluate the impact on mass transfer efficiencies. Exploring elevated system pressures which should improve mass transfer requires use of vessels made of steel or high strength plastic, rather than the standard glass vessels provided with off-the-shelf lab scale bioreactors. Improved gas dissolution and mass transfer into solution should in turn increase productivity. Kiverdi has constructed a bioreactor of high-strength plastic, pictured in Figure 47, which is able to run at elevated pressures. It was constructed with off-the-shelf materials and components which should enable lower costs. The design and operation principles of the custom bioreactor allow scaling to larger volume without need for major system re-engineering.

Figure 47: Custom Prototype Reactor for Enhanced Gas-Lquid Transfer

It is expected that scaling up the reactor sizes, and specifically scaling-up vertically, will enhance mass transfer, through increases in hydrostatic pressure. Increased hydrostatic pressure should lead to increased dissolution of the gases. The potential benefit of increased hydrostatic pressure was not adequately explored in this project because of the low volumetric gas rates used in the larger bioreactors. The throughput of gases was greatly decreased at the 20 L scale compared to the 1 L scale bioreactors (0.1-0.2 VVM versus 1-2 VVM), because the consumption of gas supply tanks would have been impractical to handle at higher flow rates. Thus an “apples-to-apple comparison” between the 1-L and 20-L scale was not made in this project. With implementation of a gas recirculation system such that 1 to 2 VVM gas flow rates can be practically maintained at larger scales, the effects of increased hydrostatic pressure can be tested and compared. Increased conversion of gaseous reactants should also result from a lengthened period of gas-liquid contact as the gas bubbles rise through a taller liquid column. In

this project the relatively low conversion of gaseous reactants, and specifically O_2, constrained the O_2 partial pressures allowed in the input gas mixture, since a safe non-flammable gas concentration had to be insured in the headspace. More thorough conversion of the O_2 per pass through the working volume could enable the use of increased O_2 partial pressures in the input gas mixture. This in turn could enable increased flux of O_2 into solution and increased productivities. Taking these various effects into account, it is possible, that in contrast to many other biological and chemical reactions that decrease in performance with increased scale, a knallgas bioprocess might actually be enhanced with scaling-up.

Knallgas Strain Improvement

In addition to targeting reactor improvements, knallgas strain improvements are also under investigation. Strains and bioprocesses are being developed that couple a high growth rate on CO_2, such as was observed in this project for *C. necator*, with greater accumulation of lipids, which can be extracted to produce oils. An additional characteristic to screen for is high energy efficiency in the conversion of H_2 and CO_2 to cell mass. Bioprocesses for growing promising knallgas strains selected through screening are being optimized over standard growth parameters such as media composition, pH, temperature, gas composition and uptake rate. The conditions (or range of conditions) that give the best growth and oil productivity are being used as the basis for end-to-end CO_2-to-oil process improvement.

Two ways of improving the amount of lipid content, generally, are being pursued: screening and selection of naturally higher lipid producing knallgas strains; and genetic engineering of knallgas strains for enhanced lipid production. Genetic engineering techniques and methods, which have been successfully applied in algae, can be also be tested in knallgas strains. Such techniques and methods include: increasing the expression of genes that encode components of the carbon-concentrating mechanism, normally induced only under low CO_2 conditions - reported to increase biomass productivities by 50 to 80 percent in algae[60, 61]; and increasing the expression of genes in the fatty acid biosynthesis pathway – reported to increase lipid content in transformed algae to over 50 percent by weight[62, 63]. A metabolic network model of a host knallgas strain such as *C. necator* can be constructed to guide further oil synthesis pathway flux optimization; such as optimization of the fatty acid biosynthesis pathway, or an isoprenoid pathway to target terpenes. An initial metabolic model can be constructed using genome information, homologous models, and expression profiling. The model can be iteratively improved with additional experimental work, such as ^{13}C flux analysis.

In addition to enhancing the lipid production and accumulation in knallgas strains through strain selection and/or genetic modification, in the short term, there are options for chemically transforming the entire biomass product (lipid and non-lipid) into biofuels.[64, 65] Such chemical conversions could be examined in parallel with strain development. However, all else being equal, this path is probably less desirable due to increased process steps, capital costs, and complexity.

The knallgas strains will also likely need to be adapted the source of CO_2 and/or syngas. This is because waste CO_2 sources such as industrial flue gases, as well as syngas generated from various feedstocks through gasification or steam reforming, may have chemical impurities that

can be inhibitory or toxic to some microorganisms. Thus the system of feedstock, gas generator, and knallgas strain should be co-designed to minimize pre-conditioning, cleanup, and processing that needs to be done to the gas before it is added to the bioreactor. As necessary, a knallgas strain could be adapted to the contaminant profile of a particular syngas, tailgas, or fluegas stream. The knallgas microorganisms used in this project have been tested on various impurities typically found in syngas and industrial flue gases. Certain strains have been identified having high tolerance to many of the contaminants present in syngas. Kiverdi is working on further increasing tolerance to residual tars and trace chemicals found in syngas, industrial tail gases, and flue gases. Synthetic syngases can be purchased to test the sensitivity of candidate strains to specific syngas contaminants such as H_2S, ethylene, other hydrocarbons, and HCl. Testing can also be performed on raw versus 'cleaned' syngas produced from a gasifier to evaluate inhibition. Impurity-tolerant knallgas strains and bioprocesses could have an additional benefit of preventing contamination because the presence of the syngas impurities would create a highly selective environment for the tolerant strain.

7.2.3 Process Integration

Optimization and scaling-up of the gas culturing, cell harvest, lipid extraction, and purification steps that were demonstrated at lab scale in this project is ongoing. Industrial scale equipment and process units will be evaluated for the downstream cell harvest, lipid extraction, and product purification process steps that are commonly used for the recovery of biological oils from oleaginous algae or yeast. In addition, work is planned on integrating the gas-to-oil process with an onsite gasifier or steam reformer; in order to supply the bioprocess with an industrial scale gaseous input.

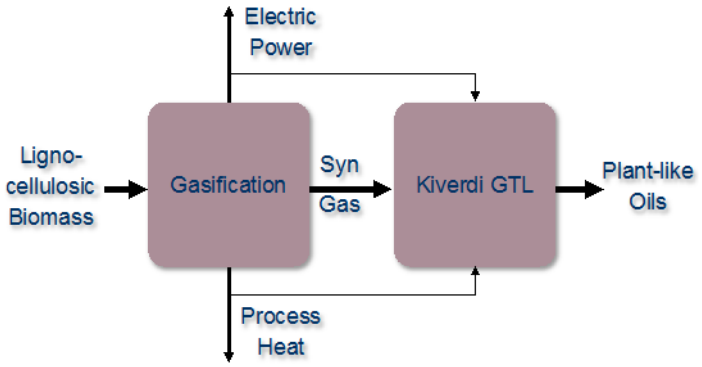
System design, such as integration of the bioconversion with the H_2/CO_2 or syngas generation step, is being studied. Important aspects of system integration include fully exploiting opportunities for heat integration by, for example, utilizing the heat co-product of syngas generation (e.g. gasification or steam reforming) in order to meet bioprocess heat or cooling demands. Examples of bioprocess heat demand that could be met using co-generated heat from a gasifier or steam reformer include biomass drying, and solvent distillation and condensation for solvent recovery and reuse in the oil extraction step. This type of system integration will enhance the economics and environmental footprint of the bioprocess, as well as the adjoining H_2 or syngas generation process. Similar to heat integration, the possibility exists of meeting the bioprocess electrical requirements. Electricity can be generated as a co-product of the H_2 or syngas generation step and utilized in some process steps. The potential for arbitrage of the various products that can be made through the renewable H_2 or syngas generation step also exists, and is an opportunity to optimize overall system economics.

An example of such an optimal systems and arbitrage approach would be generating electricity to feed into the electrical grid during peak electrical demand, and then generating H_2 used by the bioprocess for CO_2 capture and conversion during off-peak hours. Since stationary, onsite storage of H_2 is generally cheaper and simpler than the storage of electricity, stored H_2 that is generated during the off-peak hours could be used to provide continuous operation of the

bioprocess and CO_2 capture during the peak hours of electrical demand when the energy source is directed into electricity generation instead of H_2 or syngas generation.

A schematic example of a system design is illustrated in Figure 48 for the bioconversion process integrated with a gasifier for the provision of syngas generated from a lignocellulosic feedstock along with process heat and electricity. Gasification of biomass to produce syngas generally can also provide process heat and electric power coproducts, a portion of which can be used to meet the downstream bioprocess demands. Heat exchanged in cooling down the syngas prior to introduction to the bioreactor can be used as process heat, or to generate a steam and/or electricity co-product, in order to maximize the overall process efficiency and economics. Additionally, the chain of steps leading from the original energy and carbon feedstock source including harvest, delivery, storage, and pre-gasification handling and processing should be considered for process optimization. Such a systems integration approach will contribute further to the overall viability and profitability of the process described in this report.

Figure 48: System Design Integrating a Gasifier with the Oil Production Process



7.3 Final Remarks

At the commencement of this Energy Commission funded project, the technology was at a nascent stage where the basic scientific principles upon which the technology is based had been observed and a technological application had been formulated and modeled. The project enabled extensive testing and evaluation of the technology, and demonstrated function for all the components of a knallgas CO_2-to-oil bioprocess. The next step is process and strain improvement to reach desired performance in terms of productivity and yield of targeted products; and further integration of all the steps in the end-to-end CO_2-to-oil process. To translate this technology into one that can produce market-ready oil and oil-based products, Kiverdi's ongoing innovation effort is focused on: 1) strain improvement and metabolic engineering of the CO_2-to-oil pathway to make 'targeted oils' per the market and customer requirements and 2) bio-process development to further optimize and improve the low-temperature, low-pressure process into one that can produce target oil molecules commercially, at a low costs competitive with existing supplies. If the proposed system for converting CO_2 into oils can be further optimized and then scaled up economically, it can contribute significantly to the production of oils and other valuable chemicals from CO_2 waste, thereby contributing to

CO_2 mitigation by providing for large scale beneficial uses of CO_2. The proposed system can also contribute to the reduction of GHG emissions and environmental harm through the replacement of petroleum derived chemicals and fuels, and through the replacement of biological oils and oleochemicals produced through unsustainable agricultural practices such as palm oil.

GLOSSARY

Term	Definition
Acetyl-CoA	Acetyl coenzyme A is an important molecule in the metabolism of living organisms. Among many different biological roles, it is the precursor for fatty acid synthesis, terpenoid synthesis through the mevalonate pathway, and PHB synthesis.
Aerobic	An environment where O_2 is present and/or a process where O_2 is involved.
Amino acid	A molecule containing both an amine group and a carboxyl group that are bound to a carbon, which is designated the α-carbon. The basic building block of proteins.
Anabolic biosynthesis	Metabolic pathways that consume energy in the conversion of reactants into products. Usually, (but not always), the metabolic pathways that construct larger molecules from smaller units are anabolic pathways. An example is the biological production of fatty acids starting with the metabolic C2 intermediate acetyl-CoA driven by ATP. The energy consumed by anabolic biosynthesis is generally provided by respiration.
Anaerobic	An environment where O_2 is absent and/or a process where O_2 is not involved.
ATP	Adenosine-5'-triphosphate is a nucleoside triphosphate used as an intracellular carrier of chemical potential energy. It is sometimes called the energy currency of life.
Biodiesel	Biologically produced fatty acid alkyl ester suitable for use as a fuel in a diesel engine.
Biomass	The material produced by living organisms and by propagation of cells. Biomass may contain cells and intracellular contents as well as extracellular substances, including compounds secreted or excreted by a cell. In this project, the relevant biomass is the living and dead organic material composing the Knallgas microorganisms. In the context of potential feedstock for the production of H_2 and CO_2 via gasification, biomass includes agricultural and forestry residues; food waste; purpose grown crops; sewage; dung; wood waste collected in hazardous fuel reduction treatments for forest fire prevention, or generated at lumber mills and woodshops, or other types and sources of wood or saw dust waste.

Bioreactor	A closed or partially closed vessel suitable for growing or maintaining microorganisms. An example would be a fermentation system for producing beer. More complex examples would be closed vessels having controls for input gases, temperature, agitation, filtration, pressure, and so forth such that the growth of microorganisms can be optimized and contained. A bioreactor will have a supply of inputs, and outputs so that raw materials and an inoculant can be introduced, growth controlled, then the biological products can be removed for further processing. The cells may be, but are not necessarily held in liquid suspension. In some examples rather than being held in liquid suspension, cells may alternatively be growing and/or maintained in contact with, on, or within another non-liquid substrate such as a solid growth support material. Bioreactors can be configured for batch or continuous production, or mixed-modes where some material can be retained between batches as inoculant. Due to the growth of the biotechnology industry, high quality bioreactors of many sizes and configurations are readily available from commercial suppliers and comply with industry standards.
Carboxydotroph	Carbon monoxide (CO) utilizing microorganism. CO is used in the organism's metabolism as an electron donor and/or as a carbon source.
Catalyst	A chemical actor, such as a molecule or macromolecular structure, which accelerates the speed at which a chemical reaction occurs where a reactant or reactants is converted into a product or products, while the catalyst is not turned into a product itself, or otherwise changed or consumed at the completion of the chemical reaction. After a catalyst participates in one chemical reaction, because it is unchanged, it may participate in further chemical reactions, acting on additional reactants to create additional products. To accelerate a chemical reaction a catalyst decreases the activation energy barrier across the reaction path allowing it to occur at a colder temperature, or faster at a given temperature. In this way a more rapid approach of the system to chemical equilibrium may be achieved. Catalysts subsume enzymes, which are protein catalysts.
Cellulosic	Any material with a high amount of cellulose, which is a polysaccharide having the formula $(C_6H_{10}O_5)_n$, that generally consists of a linear chain of hundreds to thousands of $\beta(1\rightarrow4)$ linked D-glucose monomers. Sources of cellulosic material include but are not limited to cardboard, cotton, corn stover, paper, lumber

	chips, sawdust, sugar beet pulp, sugar cane bagasses, and switchgrass.
Chemoautotrophic	Type of microorganism that uses inorganic chemical potential energy to drive the biochemical conversion of CO_2, instead of radiant energy as in photosynthetic algae and plants (i.e. photoautotrophs).
CoA	Coenzyme A, or CoA for short, refers to an organic cofactor for condensing enzymes involved in fatty acid synthesis and oxidation, pyruvate oxidation, acetyl or other acyl group transfer, and in other acetylation.
CODH	Carbon Monoxide Dehydrogenase. An enzyme that catalyzes the conversion of carbon monoxide with water and an electron acceptor substrate to produce carbon dioxide and the reduced acceptor. Examples of reactions catalyzed by CODH type enzymes include an intracellular water-gas shift reaction converting CO and water to CO_2 and H_2: $CO + H_2O \rightarrow H_2 + CO_2$ Alternatively, some versions of CODH catalyze the reduction of the intracellular electron acceptor NAD^+, to produce the intracellular electron carrier and donor NADH, as follows: CO + H2O + NAD+ -> CO2 + NADH + H+ Microorganisms with CODH are typically carboxydotrophs. CODH provides an important step in the global Carbon Cycle.
Cofactor	Subsumes all molecules needed by an enzyme to perform its catalytic activity.
CSTR	Continuous-flow stirred-reactor. A stirred tank reactor, where, when the reactor is behaving in an ideal manner, there is total back-mixing, and the outlet product stream is identical with the bulk liquid phase within the reactor volume, and constant over time.
Cupriavidus necator (C. necator)	A Knallgas micro-organism having robust growth properties and showing great promise for bioconversion.
Dilution rate	In a continuous flow process, the rate of flow of medium into the reactor divided by volume in the reactor.
DO	Dissolved oxygen – the amount of oxygen in solution (aqueous) phase. Typically stated as the amount relative to saturation under a given condition.

Electrolysis	In chemistry and manufacturing, electrolysis is a technique that uses a direct electric current (DC) to drive an otherwise non-spontaneous chemical reaction. In the context of this report electrolysis refers to the electrolysis of water or brine to produce H_2. Electrolysis of water is described by the following formula $2H_2O_{(l)} \rightarrow 2H_{2(g)} + O_{2(g)}$; E_0 = -1.229 V
Electron acceptor	Chemical entity that accepts electrons from an electron donor in a redox reaction. It is an oxidizing agent that, by virtue of accepting electrons, is itself reduced in the process. Examples of electron acceptors are O_2 and CO_2.
Electron donor	Chemical entity that donates electrons to an electron acceptor in a redox reaction. It is a reducing agent that, by virtue of donating electrons, is itself oxidized in the process. Examples of electron donors are H_2 and CO.
Exogenous gene	A nucleic acid that has been recombinantly introduced into a cell, which encodes the synthesis of RNA and/or protein. The exogenous gene may be introduced by transformation. An exogenous gene may be introduced into a cell by electroporation. A transformed cell may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. The exogenous gene put into the host species may be taken from a different species (this is called heterologous), or it may naturally occur within the same species (this is homologous as defined below). Therefore, exogenous genes subsume homologous genes that are integrated within or introduced to regions of the genome, episome, or plasmid that differ from the locations where the gene naturally occurs. Multiple copies of the exogenous gene may be introduced into the cell. An exogenous gene may be present in more than one copy within the host cell or transformed cell.
FAD	Fatty aldehyde decarbonylase (FAD) refers to an enzyme catalyzing the reaction that produces an alkane from a fatty aldehyde molecule by decarbonylation.
FadR	Fatty acyl-ACP/acyl-CoA reductase. Refers to an enzyme catalyzing the reaction that produces a fatty aldehyde from an acyl-ACP or acyl-CoA molecule by reduction.
Feedstock	Raw material for a biological, chemical, or other industrial process, which is converted into finished products by the process.
F-T synthesis; Fischer-	A chemical process to create middle distillates such as jet fuel and diesel (C8-C18) from syngas, as well as longer carbon chain lengths

Tropsch Process	extending out to heavy waxes (C30+) from syngas. F-T is a commercial scale, proven process.
Gas chromatography/mass spectrometry (GC/MS)	A chemical analytical method combining Gas Chromatography and Mass Spectrometry. Gas Chromatography separates chemicals present in a mixture (generally by molecular weight and adsorption properties) so that components of the mixture can be identified. Each of these components is then subjected to Mass Spectrometry, which decomposes each chemical into atoms so that the empirical chemical formula of that chemical can be determined. This method is a powerful tool for analyzing and identifying the composition of chemicals in a mixture.
Gas Headspace	The gas volume in a bioreactor above a liquid phase where the biological production is occurring. This space can be used to measure (providing information about reaction conditions) or control gas composition (potentially altering reaction conditions). The headspace may be subject to phenomenon such as foaming that may be diagnostic relative to grown conditions and which may need active control measures.
Gasification	Refers to a generally high temperature (>700°C) process that converts carbonaceous or carbon-based materials into a mixture of gases including hydrogen, carbon monoxide, and carbon dioxide called syngas or producer gas. The process generally involves partial combustion and/or the application of externally generated heat along with the controlled addition of oxygen and/or steam. O_2 is kept limited so that complete combustion to CO_2 and H_2O is prevented.
Hydrocarbon	A molecule composed exclusively of carbon and hydrogen atoms with the carbons bonded covalently in a branched or linear chain, or cyclic ring, or rings, and with hydrogen atoms covalently bonded to the carbons such that the chemical octet rule for the carbons is generally satisfied. In some hydrocarbons there may occur some number of double or triple bonds between adjacent carbon atoms in the chain. Thus, the label hydrocarbon subsumes branched, cyclic, or linear alkanes (also called paraffins), alkenes (also called olefins), alkynes, and aromatics. The structure of hydrocarbon molecules range from the smallest, methane (CH4), a primary component of natural gas, to high molecular weight complex molecules including asphaltenes present in bitumens crude oil, and petroleum. Hydrocarbons may occur in gaseous, liquid, or solid phases, either singly, or in multiply coexisting

	phases.
Hydrogenase	An enzyme that catalyzes the oxidation of molecular hydrogen (H_2). H_2 oxidation is generally coupled to the reduction of an electron acceptor or carrier such as NAD^+ as described by the reaction $H_2 + NAD^+ \rightarrow NADH + H^+$. Hydrogenases are generally present in hydrogenotrophs.
Hydrogenotroph	A H_2-oxidising chemoautotroph wherein the electron donor in the chemoautotrophic reaction is H_2.
Homologous	Sequences homology between two nucleic acid sequences or two amino acid sequences. Two nucleic acid sequences or two amino acid sequences that are sufficiently homologous to retain immunogenic function are "homologues."
Inoculum	A sample of material (usually an aqueous solution) containing a desired microorganism used to seed a medium in a bioreactor so that the desired microorganism can be cultivated
Isoprenoid pathway	The metabolic pathway to isoprenoids, also called terpenoids, and terpenes. These products of isoprenoid pathways represent a large and diverse class of lipids and non-saponifiable oils derived from five-carbon isoprene units assembled and modified in a multitude of ways, generally resulting in cyclic or branched molecular structures. These lipids and oils can be found in all classes of living things, and are the largest group of natural products. About 60% of known natural products are isoprenoids or terpenes. Examples include limonene and squalene. Commercial products composed isoprenoids or terpenes include citrus or orange oil and turpentine.
Jet fuel	A fuel useful for igniting in the engine of an aircraft comprising a mixture of kerosene combined with typical additives. Straight-run kerosene, the basic component of the kerosene used for jet fuels, consists of hydrocarbons with carbon numbers mostly in the C9–C16 range. Like all jet fuels, straight-run kerosene consists of a complex mixture of aliphatic and aromatic hydrocarbons. Aliphatic alkanes (paraffins) and cycloalkanes (naphthenes) are hydrogen saturated, clean burning, and chemically stable and together constitute the major part of kerosene. Jet fuel may comprise a mixture of ingredients specified by the Jet A-1, Jet A, Jet B, JP1, JP-2, JP-3, JP-4, JP-5, JP-6, JP-7, JP-8, JP-10 or other similar compositions.
Knallgas	A mixture of hydrogen (H_2) and oxygen (O_2) gases
Knallgas	A microbe that can use hydrogen as an electron donor and oxygen

microorganism	as an electron acceptor in the generation of intracellular energy carriers such as Adenosine-5'-triphosphate (ATP). The terms "oxyhydrogen" and "oxyhydrogen microorganism" can be used synonymously with "knallgas" and "knallgas microorganism" respectively.
Lignocellulosic material	Any material composed of cellulose, hemicellulose, and lignin where the carbohydrate polymers (cellulose and hemicelluloses) are tightly bound to lignin. Lignocellulosic materials subsume agricultural residues (including corn stover and sugarcane bagasse), most biomass energy crops, wood residues (including sawmill and paper mill discards), and a substantial fraction of municipal waste.
Lipid	A category of biologically synthesized molecules that can be dissolved in nonpolar solvents (such as chloroform and/or ether and/or hexane) and which also have low or no solubility in water. The hydrophobic character of lipids molecules typically results from the presence of long chain hydrocarbon sections within the molecule. Lipids subsume the following molecule types: biologically synthesized hydrocarbons, fatty acids (saturated and unsaturated), fatty alcohols, fatty aldehydes, hydroxy acids, diacids, monoglycerides, diglycerides, triglycerides, phospholipids, sphingolipids, sterols such as cholesterol and steroid hormones, fat-soluble vitamins (such as vitamins A, D, E and K), polyketides, terpenoids, terpenes, and waxes.
Microorganism or microbe	Single celled life forms.
Mineral Salt Medium (MSM)	A salt solution, used in this project as a starting point for creating an aqueous growth environment for Knallgas microorganisms, containing no organic carbon or complex nutrients
Molecule	Any distinct or distinguishable structural unit of matter comprising one or more atoms, and includes for example hydrocarbons, lipids, polypeptides and polynucleotides.
MSR	Methane steam reforming reaction. Alternatively steam methane reforming (SMR). The most common method of producing commercial bulk H_2. The H_2 is often used in the industrial synthesis of ammonia, urea, and other chemicals or in the hydrotreatment of crude oils. MSR is also used to produce syngas for commercial scale F-T. At high temperatures (700 – 1100 °C) and in the presence of a metal-based catalyst, steam reacts with methane to yield

	carbon monoxide and H_2. $CH_4 + H_2O \rightleftharpoons CO + 3H_2$ Additional hydrogen can be recovered by following MSR with a lower-temperature water gas-shift reaction to convert the carbon monoxide produced in the MSR with water to produce additional hydrogen. This reaction is summarized by: $CO + H_2O \rightleftharpoons CO_2 + H_2$ The net combined reaction of MSR followed by the water gas shift reaction is $CH_4 + 2H_2O \rightleftharpoons CO_2 + 4H_2$ This combination of reactions is generally performed currently in the commercial bulk production of H_2. When a pure H_2 stream is desired, such as for ammonia production through the Haber-Bosch process at fertilizer factories, or for hydrotreatment of crude petroleum at oil refineries, then a final CO_2 capture step is performed to remove the CO_2 from the gas mixture, leaving H_2 as the product.
MTG	Methanol-to-gasoline process. Developed by Mobil in the early 1970s where methanol is polymerized over a zeolite catalyst to form gasoline-type hydrocarbons including branched, cyclic, and aromatic molecular structures.
NADH	Nicotinamide adenine dinucleotide hydride. A coenzyme involved in intracellular redox reactions - carrying electrons from one reaction to another. It is found in two states inside cells. NAD^+ is the oxidized state. NAD^+ acts as an oxidizing agent, accepting electrons from other molecules (e.g. H_2), entering its reduced state NADH. NADH acts as a reducing agent, donating electrons to other molecules (e.g. CO_2).
NADPH	Nicotinamide adenine dinucleotide phosphate hydride. A coenzyme very similar to NADH involved in intracellular redox reactions - carrying electrons from one reaction to another. It differs from NADH in the presence of an additional phosphate group on the 2′ position of the ribose ring that carries the adenine moiety. NADH and NADPH are very close in redox potential.
Nutrient Amendment	Supplementation of input nutrients during a growth run in a bioreactor to alter or prolong growth of microorganisms. Often determined in response to an indirect measurement of biomass production such as OD measurement of a sample taken from a

	bioreactor, a pH measurement or a gas concentration measurement.
OD	Optical density – a measurement of how much light is blocked by suspended solids in a fluid sample. OD can be used to indirectly measure the amount of solid materials, such as the cell material of organisms, which are present in a fluid sample.
Oils Extraction	The process by which various oils and fats are separated from the general biomass produced by a bioreactor run. Typically, extraction includes both mechanical and chemical means. For example, materials may be mixed with a solvent and subjected to high g forces in a centrifuge. Depending on what material is targeted and the purity required, extraction may be simple or relatively complex. Typical solvents used include hexane and mixtures of chloroform and methanol.
Oleaginous	Something that is rich in oil and/or lipids or produces oil and/or lipids in high quantities.
Oleochemical	Chemicals derived from biological oils or fats. Currently oil or fats from plants or animals are primarily used for the production of oleochemicals. They are analogous to petrochemicals derived from petroleum.
Organic molecule, chemical, or compound	Any gaseous, liquid, or solid chemical compounds which contain carbon atoms except the following exceptions, which are considered inorganic: carbides, carbonates, simple oxides of carbon, cyanides, and allotropes of pure carbon such as diamond and graphite.
Oxidation	The loss of electrons, or in other words, an increase in oxidation state, of a molecule, atom, or ion. An "oxidizing agent" causes oxidation of another chemical entity. For example O_2 is an oxidizing agent that causes oxidation of H_2 or CO.
PHB	Poly-hydroxybutyrate, a polyester biopolymer produced by bacterial microorganisms.
Photoautotrophic	Type of microorganism that uses radiant energy to convert CO_2 to grow. Typical examples are photosynthetic algae and plants.
Promoter	A control DNA sequence that regulates transcription. A promoter may include nucleic acid sequences near the start site of transcription that are required for proper function of the promoter, as for example, a TATA element for a promoter of polymerase II type. The term "inducible promoter" refers to an operable linkage

	between a promoter and a nucleic acid where the promoter's mediation of nucleic acid transcription is sensitive to a specific stimulus. An "operable linkage" refers to an operative connection between nucleic acid sequences, such as for example between a control sequence (e.g. a promoter) and another sequence that codes for a protein i.e. a coding sequence. If a promoter can regulate transcription of an exogenous gene then it is in operable linkage with the gene.
Redox reaction	Chemical reactions in which atoms have their oxidation state changed. Generally redox reactions involve the transfer of electrons between species. The term redox comes from the two aspects of electron transfer: reduction and oxidation.
Reducing equivalent	Intracellular reducing agent. Intracellular electron carrier and donor. An example is NADH.
Reduction	The gain of electrons, or in other words, a decrease or reduction in oxidation state, of a molecule, atom, or ion. A "reducing agent" causes reduction of another chemical entity. For example H_2 is a reducing agent that causes reduction of O_2 or CO_2.
Respiration	A process in living organisms involving the production of energy for cellular processes and anabolic biosynthesis. Generally respiration involves the reaction of an electron donor, i.e. reducing agent, with an electron acceptor, i.e. oxidizing agent. An example is the knallgas reaction of H_2 and O_2. The energy released in this redox reaction is generally captured for utilization in the form of intracellular energy carriers such as ATP.
Saponifiable	A lipid with ester functional groups bonding fatty acids, which can be hydrolyzed under basic conditions. Hydrolysis under basic conditions releases the salt of the fatty acid. Examples of saponifiable lipids include TAGs and Phosphatidylcholine. Examples of non-saponifiable lipids include terpenes such as limonene or squalene.
Standard Operating Procedures (SOPs)	In this project, standard operating procedures generally refers to the medium composition, the operational conditions (pH, temperature, and the gas flow rate, gas composition), protocols for system inoculation, sampling, harvesting and analysis of product in the context of growing Knallgas organisms and producing products from CO_2. These SOPs provide baseline conditions to reliably reproduce high-efficiency growth of the target microorganisms and synthesis, recovery, and purification of

	products.
Steam reforming	A method for producing H_2, carbon monoxide, and other gaseous co-products from liquid or gaseous hydrocarbon fuels such as natural gas. This is achieved in a processing device called a reformer which reacts steam at high temperature with the hydrocarbon fuel. Often followed by the water gas shift to convert CO content into additional H_2 yield.
Syngas or Synthesis gas	Gas mixture containing H_2 and CO typically produced from gasification or steam reforming processes. May refer to the gas mixture immediately exiting a gasifier or steam reformer, which, in addition to H_2 and CO, also typically contains CO_2. This raw gas mixture also typically includes a variety of impurities such as methane, hydrogen sulfide, condensable gases, and tars. The CO_2 and impurities are generally cleaned from the syngas prior to feeding into chemical reactions such as F-T, methanol, or ammonia synthesis. "Producer gas" is a related term that generally refers to gas mixes similar to syngas except for the presence of a large N_2 component that results from using air directly in the gasification or partial oxidation process. The term "water gas" is also sometimes used to refer to a mixture of H_2 and CO.
TAG	Triacylglyceride. A fat containing three fatty acid chains attached to the hydroxyl groups of a glycerol backbone by ester bonds.
Thioesterase	A thioesterase (TE), or more specifically a fatty acyl-ACP thioesterase means an enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP) during lipid synthesis.
Turbidostat	A continuous culture system having feedback between the turbidity of the culture vessel and the dilution rate.
Water-gas shift reaction (WGSR)	The reaction of carbon monoxide and water vapor to form carbon dioxide and molecular hydrogen i.e. $CO + H_2O \rightarrow H_2 + CO_2$. A mixture of H2 and CO is sometimes referred to as "water-gas". The WGSR shifts the composition of water-gas towards more H2 and less CO. The WGSR is performed commercially on large scales at oil refineries in the production of H_2 used for petroleum hydrotreatment and at fertilizer factories to provide H_2 for ammonia and urea synthesis through the Haber-Bosch process. At elevated temperatures and excess H_2 and CO_2 the endothermic reverse water-gas shift reaction can be performed; whereby H_2 reacts with CO_2 to produce CO and the water-gas shift reaction runs in reverse i.e. $H_2 + CO_2 \rightarrow CO + H_2O$

	The reverse WGSR has been proposed as a means of converting CO_2 to CO so that it can be fed into the F-T process for production of diesel or aviation fuel from CO_2.
Wild type	A natural strain meaning a strain that has not had exogenous genes encoded into it

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[29] $NaHCO_3$ 0.5g/L

[30] $ZnSO_4 \cdot 7H_2O$ 100 μg/L, $MnCl_2 \cdot 4\ H_2O$ 30 μg/L, H_3BO_3 300 μg/L, $CoCl_2 \cdot 6H_2O$ 200 μg/L, $CuCl_2 \cdot 2H_2O$ 10 μg/L, $NiCl_2 \cdot 6H_2O$ 20 μg/L, $Na_2MoO_4 \cdot 2H_2O$ 30 μg/L

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APPENDIX A:
Observations and Comments from 20 L Pilot Scale Runs of *C. necator*

Batch	Comments / Issues / Improvements / Lessons Learned
1	This is the first batch run with *C. necator*. Successfully demonstrated growth to OD of 28, which was still increasing at the time the batch ended.
2	Demonstrated the organism was able to switch from non-active growth phase to active growth phase with minimal lag time, since this batch was inoculated on Monday from the previous batch, to which the gas supply had been stopped on the previous Friday. Experienced some issues with gas delivery resulting from a partially clogged sparger, low gas supply pressures, and one of the MFCs was not working well at these gas flows which were at its lower range.
3	The sparger was not used during this run due to being clogged. Instead, gas was input in through a 1/4" tube to the bottom of the reactor. To account for the lack of a sparger and to reduce the bubble size the stirring rate was increased during this batch. The shear insensitivity of *C. necator* was demonstrated with its ability to handle higher stirring rates and still grow. Overheating of the reactor (7 °C higher than the setpoint) occurred as a result of insufficient heat removal through the reactor walls, warmer laboratory temperatures resulting from warm weather, and heating due to high stirring rates. This suggested that passive cooling through the reactor walls may be insufficient at larger scales and that active cooling may be required. The higher temperatures may have negatively impacted the growth.
4	This was the first batch run in 20-liter reactors. Successfully demonstrated growth up to an OD of 42, which was still increasing at the time the batch was stopped, at the 20 liter scale. Observed elevated temperatures at higher stirring rates >700 rpm and thus reduced stirring rates as needed to avoid overheating. Passive cooling through reactor walls was found to be insufficient, active cooling with cooling water was needed.
5	This batch was the first batch grown in the second 20-liter reactor, which had a stirring motor limited to 200 rpm. (This motor was replaced for the next batch run in this reactor.) This lower stirring rate was found to be insufficient to provide a recycle of the headspace gases and resulted in slower growth. This was the last batch to be grown from the original inoculum.
6	Installed a cooling coil in the reactor to actively cool the reactor with cooling water to avoid overheating at higher stirring rates. Inoculated with a new inoculum that was grown on sugar. Successfully demonstrated the ability of *C. necator* to switch from sugar growth to gas growth in reactors even at a low starting OD of 0.085. This batch had significant foaming that filled the overflow reservoir. It was found that manual addition of anti-foam was insufficient for controlling foam overnight.

7	Installed a new stirring motor on reactor B that allowed for increased stirring rate. The top impeller was larger than the one in reactor A that allowed for increased mixing at lower stirring rates. A cooling coil was also added to actively cool the reactor to allow higher stirring rates. Over the course of the batch the stirring rate was increased stepwise in order to observe the performance the new motor in stages, rather than set it to a higher stirring rate initially. A media composition with higher Fe content was attempted to reduce foaming; however there was no apparent foam reduction. Over the course of the batch ~50 mL of antifoam was added automatically.
8	A media composition with higher Fe content was tested to reduce foaming; however there was no apparent foam reduction. Over the course of the batch ~50 mL of antifoam was added automatically.
9	Continued to use media with higher Fe content in attempts to reduce foaming, however there was no apparent foam reduction. The size of the tubing in the antifoam delivery system was reduced to reduce antifoam delivery. ~25 mL of antifoam was added automatically during the course of the batch. 50 mL samples were collected daily, and centrifuged for lipid analysis.
10	Continued to use media with higher Fe content in attempts to reduce foaming, however there was no apparent foam reduction. Reduced the size of the tubing to reduce antifoam delivery. ~42 mL of antifoam was added automatically during the course of the batch. 50 mL samples were collected daily and centrifuged for lipid analysis.
12	Instead of using anti-foam, adjusted the gas flow rates and stirring rates to manage the foaming in the headspace and avoid over flowing the reactor. This resulted in many changes to the gas flow rates and stirring rates, while an attempt was made to both maximize growth and manage the foam. It appeared that the inlet gas flow had a bigger effect to foaming over the reactor rather than the stirring rate in this reactor configuration.
13	Inoculated this batch with *C. necator* (S1) that was growing concurrently in batch 12. Instead of using anti-foam, adjusted the gas flow rates and stirring rates to manage the foaming in the headspace and avoid over flowing the reactor. It appeared that the inlet gas flow had a bigger effect on foaming over the reactor rather than the stirring rate in this reactor configuration.

APPENDIX B:
Pilot Plant Process Flow Diagram and Layout

Figure B.1: Process Flow Diagram for 50 L Mobile Pilot Plant

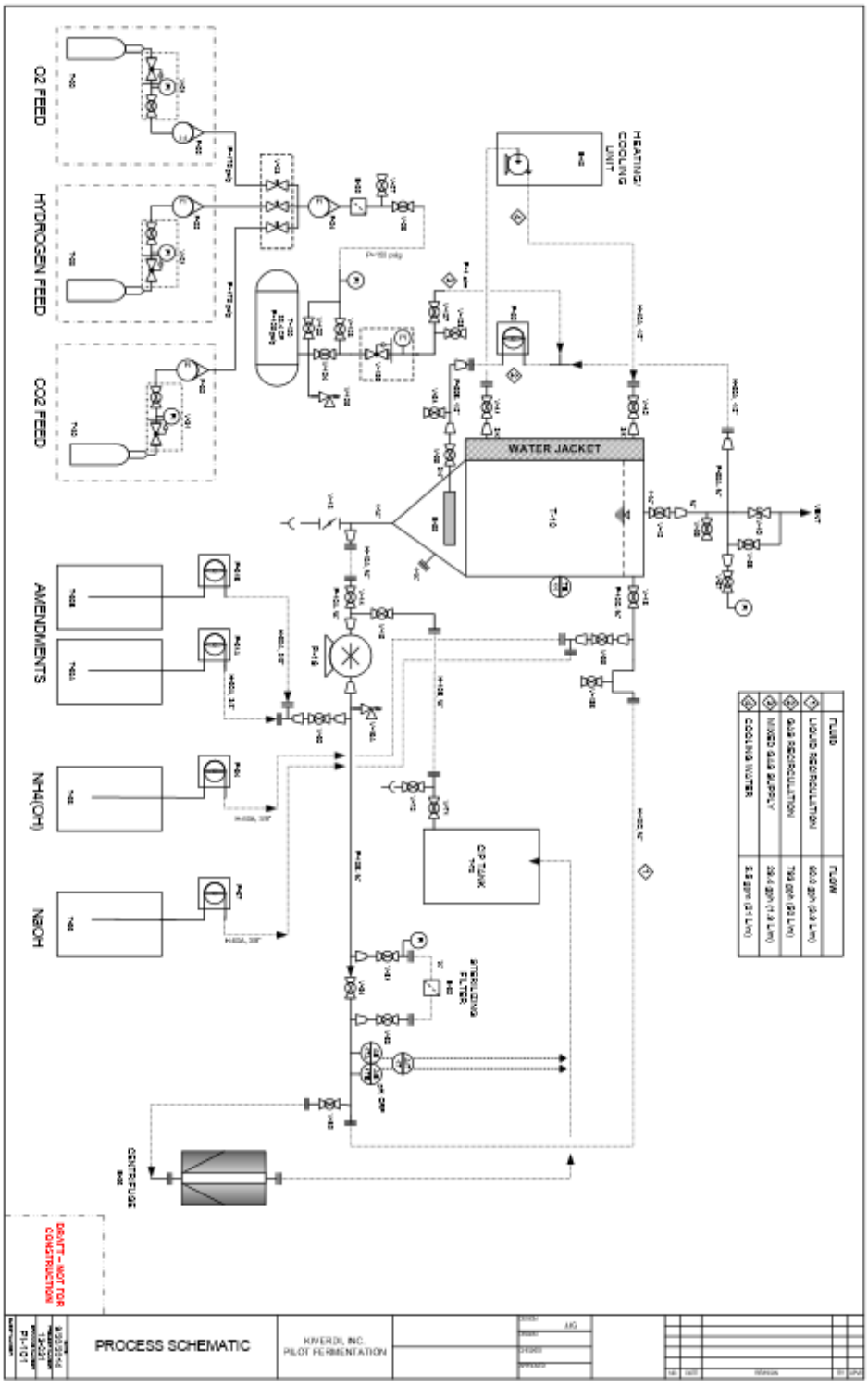

Figure B.2: Pilot Plant Layout inside Shipping Container.